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Chromatographic Determination of Evans Blue in Plasma and Serum

By C. J. O. R. MORRIS (Freedom Research Fellow), *Clinical Laboratory and Medical Unit, the London Hospital*

(*Received* 11 *May* 1944)

The methods at present available for the determination of the dye, Evans blue, in plasma have certain disadvantages. Direct colorimetry of dyed plasma is inapplicable where the plasma is haemolyzed, turbid or lipaemic. The butanol extraction procedure of Harington, Pochin & Squire (1940) is troublesome and time-consuming where many analyses must be carried out; its accuracy is lower than those of other methods (Bowler, Crooke & Morris, 1944); it is liable to interference by haemolysis; and the modification suggested for haemolyzed plasma has not proved satisfactory in this laboratory. The method of Crooke & Morris (1942), which involves precipitation of plasma proteins and pigments with a hydrochloric acid-ethanolic phosphotungstic acid mixture, also has its disadvantages. It is likewise liable to interference by haemolysis, although this can be obviated by an optical correction method (Morris, 1944); and in certain specimens of phosphotungstic acid there is a substance which brings about the photochemical oxidation of Evans blue to a colourless compound. This difficulty can be overcome by working in artificial light. Certain plasma samples do not give an absolutely clear supernatant fluid on precipitation, and the occurrence of this effect, although uncommon, cannot be predicted. In Phillips's (1943) method the effect of interfering substances is allowed for by measuring residual light absorption after the Evans blue has been reduced to a colourless compound with sodium hydrosulphide. The method gives satisfactory results in the presence of a considerable degree of haemolysis, but the reduction requires 12 hr. for completion, and a supply of pure carbon monoxide is necessary.

In the course of an investigation of the homogeneity of commercial samples of Evans blue, it was observed that the dye was adsorbed from aqueous solution by aluminium hydroxide. This suggested that it might be possible to effect a quantitative separation of the dye from plasma or serum by chromatography. The adsorption behaviour of Evans blue in solution in plasma is, however, quite different from that of the dye in aqueous solution. Evans blue is only adsorbed from plasma at approximately pH 10. At this pH it is not adsorbed from aqueous solution. Ogston (1943) has shown by means of the ultracentrifuge that Evans blue combines with the plasma proteins, and it is therefore probable that the difference in adsorption behaviour, and also certain differences in light absorption (Phillips, 1943), are due to the existence of an Evans blue protein complex. With suitable conditions, however, it is possible to separate the dye from plasma by chromatographic means, the plasma proteins and pigments passing directly through the adsorption column. If the sample is haemolyzed, a portion of the alkaline haematin formed on adjusting to pH 10 is adsorbed on the column, but this can be removed by elution with acetic acid-ethanol. The dye is subsequently eluted from the adsorbent with hydrochloric acid-ethanol and the concentration in the eluate determined colorimetrically. This method permits a complete separation of Evans blue from all other light-absorbing substances present in plasma. If it is necessary to measure very low dye concentrations, it is possible to achieve a high degree of accuracy by concentrating the dye from larger plasma samples than those usually employed.

METHODS

Reagents

Adsorbent: 1 part (by wt.) of MgO Analar and 20 parts of $Al(OH)_3$ (The British Drug Houses, Ltd. 'dry') are thoroughly mixed by shaking in a bottle.

2 N-NaOH *solution.*

Acetic acid-ethanol wash liquid: 1 vol. glacial acetic acid Analar and 9 vol. 70 % (by vol.) ethanol.

Eluent: 8 vol. water, 10 vol. conc. HCl (sp. gr. 1·18) and 32 vol. absolute ethanol.

Apparatus

A two-holed rubber bung is provided with a sintered glass micro-filter tube (porosity 2, type 232 H 2, Baird and Tatlock Ltd.) and a side tube for connexion to the water pump. 15 ml. centrifuge tubes are used as receivers.

Technique

Determination of Evans blue. The dry filter tube is filled with the adsorbent to a depth of about 2 cm. Uniform packing is ensured by tamping with a glass pestle during the filling. 1·00 ml. of dyed plasma or serum is measured into a suitable tube; 0·30 ml. of 2 N-NaOH is added and thoroughly mixed. 1·00 ml. of the mixture is pipetted on to the adsorption column and allowed to wet the adsorbent by capillary action. Gentle suction is now applied until the mixture has passed through the column. A bright blue zone forms on the upper part of the adsorbent. The column

is washed with 5 ml. of water which removes remaining plasma proteins and pigments, and with 4 ml. of the acetic acid-ethanol mixture which elutes any haem pigments present. The wash liquid is removed as completely as possible by suction, the receiver changed and the dye eluted with the HCl-ethanol mixture. The bright blue zone passes down the column and into the filtrate. About 8 ml. of eluent are necessary for complete elution. The eluate is transferred quantitatively to a 10 ml. volumetric flask and diluted to volume with the eluent mixture. The dye concentration is then determined colorimetrically.

Colour measurement

The determinations described in this work were made with a photoelectric absorptiometer constructed in this laboratory. An Ilford spectrum orange filter (no. 607, maximum transmission at 6000 A.) was used with a tungsten filament light source. Infra-red radiation was removed by a 2 mm. thickness of Chance calorex glass. Extinctions were measured in 20 mm. solution thickness. The dye concentration can then be found by reference to a standard curve. It is, however, advisable to check the concentration of the solution used for injection in plasma volume determinations by diluting 0·10 ml. of a 1 in 50 dilution of this injection solution to 10 ml. with eluent mixture and determining its extinction. This value can then be used as a standard in plasma volume determinations. All extinctions were measured against eluent mixture as control. The absence of interfering substances in the solutions to be measured, and the fact that solutions of Evans blue obey the Beer-Lambert law under the conditions used, suggests that it should be possible to obtain reliable results with a visual Dubosecq pattern colorimeter. In this case larger plasma samples (2–3 ml.) should be used to avoid the difficulty of matching low colour intensities.

RESULTS

Light absorption of Evans blue in ethanolic HCl *solution*

The values given below were obtained by diluting known quantities of dye solution to 10 ml. with eluent mixture:

Evans blue (μg.)	Extinction
7	0·105
14	0·209
28	0·409
42	0·629

It will be seen that the Beer-Lambert law is obeyed.

Recovery of Evans blue from non-haemolyzed serum

Varying amounts of Evans blue were added to non-haemolyzed serum (1 ml.) and taken through the routine process. The values (corrected for volume differences) were as follows:

Evans blue present (μg.)	Evans blue found (μg.)	Recovery (%)
7·0	6·95	99·2
14·0	14·0	100·0
28·0	28·0	100·0
42·0	41·0	97·6

It will be seen that recovery is less complete at higher dye concentrations. These levels will, however, only rarely be reached even when two plasma volume determinations are carried out in rapid succession. With the amount of dye usually injected in the course of a plasma volume determination (35 mg.) the plasma concentration is 10–15 μg./ml.

Recovery of Evans blue from haemolyzed serum

14 μg. of Evans blue were added to 1·0 ml. of non-haemolyzed serum and kept for 10 min. Varying amounts of haemoglobin were added and the mixture taken through the routine process. The results are given below. It will be seen that the recovery is independent of the amount of haemoglobin present. Determination of the extinctions of the solutions with a combination of Ilford spectrum violet and Ilford aviol filters (Morris, 1944) indicated that the amount of haem pigment present was negligible. If the haemoglobin is added to the serum before the Evans blue, it apparently combines with the dye to some extent and the haemoglobin-Evans blue complex is removed by the acetic acid-ethanol wash liquid with consequent loss of dye. It is possible that the method will not be applicable to cases where there is considerable intravascular haemolysis.

Hb present (mg.)	Evans blue present (μg.)	Evans blue found (μg.)
1·5	14·0	14·0
3·0	14·0	14·0
4·5	14·0	14·0

SUMMARY

A new method for the determination of Evans blue is described, depending on the selective adsorption of the dye on a column of aluminium hydroxide and magnesium oxide, and subsequent elution with hydrochloric acid-ethanol. The dye concentration in the eluate is measured photometrically.

The expenses of this research were defrayed from the Yarrow Fund of the London Hospital.

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Isolation of Trehalose from Desert Manna

By J. LEIBOWITZ, *Cancer Research Laboratories, Department of Chemistry, The Hebrew University, Jerusalem*

(*Received* 26 *April* 1944)

The manna found on the leaves of *Tamarix* in the Sinai Desert has been described in detail by Bodenheimer & Theodor (1929), and has been identified as the excretion of the scale insects *Trabutina mannipara* and *Najacoccus serpentinus*. Its chemical analysis by Fodor & Cohn (1929) established the presence of sucrose and invert sugar as the principal or sole sugar constituents.

Some specimens of another manna collected in the North Iraqian Desert (Suleimanya District) were kindly supplied to me by Prof. Bodenheimer, who believes that this manna, too, is the excretion of some scale insect. It is gathered by the Bedouin from the leaves of trees and bushes and is used as a source of sweetening substance.

EXPERIMENTAL

The specimens examined included two batches of the raw material, and one of the syrup which is produced from the manna. The method of preparation of the syrup from its natural source is unknown to me. It is water-clear, with a slightly yellowish colour, and is of a thick consistency; it has a pronounced sweet taste. The substance consists mainly of carbohydrate and gives only a very slight precipitate with trichloroacetic acid and other protein precipitants. The aqueous solution shows a surprisingly high dextrorotation, corresponding to a $[\alpha]_D$ of over $+100°$, which rules out the possibility that sucrose or invert sugar or any of the common hexoses is the principal constituent. The solution was analyzed by differential hydrolysis, the changes in optical activity and reducing power to Bertrand reagent and Willstätter-Schudel reagent being followed simultaneously.

The stock solution consisted of 5 g. of syrup (dried in a desiccator, but still containing some water) dissolved in 50 ml. of water. Hydrolysis was carried out on samples of this solution by heating with measured amounts of H_2SO_4 of varying concentrations, or by incubating with a solution of yeast invertase in phosphate buffer of pH 4·5. All reducing values refer to 1 ml. of the original solution or to equivalent amounts of the hydrolysates. Total reducing sugar was determined by the method of Bertrand. Aldoses were determined by the method of Willstätter & Schudel as modified by Auerbach & Bodländer (1923). Ketose was calculated as the difference between the Bertrand and the Willstätter-Schudel reducing values. All polarimetric data refer to readings in a 1 dm. tube. In the case of the hydrolysates the values are corrected for the changes in volume induced in the original solution by the treatment. The data are summarized in Table 1.

Table 1. *Changes in optical rotation and reducing power produced by hydrolysis of manna*

	Treatment of original solution	$[\alpha]_D$	Total hexose (mg.)	Aldose (mg.)	Ketose (mg.)
(1)	None	$+10·3°$	22·1	15·1	7·0
(2)	0·01 N-H_2SO_4, 30 min. at 100°	$+ 9·2°$	34·4	21·0	13·4
(3)	Invertase, 24 hr. at 37°	$+ 9·0°$	34·0	22·2	11·8
(4)	0·5 N-H_2SO_4, 60 min. at 100°	*	57·5	44·0	13·5
(5)	N-H_2SO_4, 60 min. at 100°	*	76·6	68·3	(8·3)
(6)	N-H_2SO_4, 75 min. at 100°	*	78·0	70·3	(7·7)

* Owing to a dark coloration of the solution no observation was possible.

It follows from (1) and (6) of Table 1 that about one-quarter of the carbohydrate consists of reducing sugar, apparently invert sugar with an excess of glucose. The transition from (1) to (2) marks the inversion of sucrose, identified by its susceptibility to mild hydrolysis and its conversion into an approximately equimolecular mixture of aldose and ketose. This identification is corroborated by the agreement between the data of the mild acid hydrolysis (2) and of the enzymatic hydrolysis (3), as well as by the amount of decrease in rotational power: observed for (2) $10·3 - 9·2 = 1·1°$, observed for (3) $10·3 - 9·0 = 1·3°$. Calculated for the inversion of 1·17 % sucrose, corresponding to 1·23 % invert sugar,

$$\left[66 \times \frac{1·17}{100}\right] - \left[-20 \times \frac{1·23}{100}\right] = 1·02°.$$

The bulk of the substance, however, is some non-reducing carbohydrate different from sucrose. It is remarkably resistant to acid hydrolysis and yields on ultimate hydrolysis only aldose. The apparent drop in the ketose values from (4) through (5) to (6) is caused by the prolonged action of the hot strong acid on fructose. Control experiments with invert sugar showed that under the same conditions fructose is partially destroyed, causing a dark coloration

of the solution, while the glucose component remains unaffected. By comparing (6) with (2) or (3) of Table 1, the amount of this fraction can be calculated as 60 % of the total carbohydrate, and it is obvious that its rotation must be about $[\alpha]_D = +200°$. These results suggest the identity of this fraction with trehalose. The data would have been consistent also with the presence of glycogen or starch, but this is definitely excluded by the failure of the solution to give a colour with iodine.

On the basis of this assumption it should be possible to compute the optical rotation of the original solution as follows:

1·51 % glucose (1) ...	... $[\alpha]_D =$	$52 \times \frac{1·51}{100} = +0·78°$
0·70 % fructose (1)...	... $[\alpha]_D =$	$(-92) \times \frac{0·7}{100} = -0·64°$
1·17 % sucrose (2) – (1) (≡1·23 % invert sugar)	... $[\alpha]_D =$	$66 \times \frac{1·17}{100} = +0·77°$
4·68 % trehalose (6) – (2) (≡4·93 % glucose)	... $[\alpha]_D =$	$197 \times \frac{4·68}{100} = +9·22°$
	Total $[\alpha]_D =$	$+10·13°$

The excellent agreement between this calculated value and that observed (+10·3°) strongly supports the assumptions employed in making this calculation.

To confirm the nature of the unknown sugar the following experiment was undertaken with a specimen of the natural manna. The raw material was a dark grey sticky mass containing some granular material together with leaf and insect residues. The material (30 g.) was fermented with baker's yeast until all reducing sugar (and presumably also all the sucrose) had been removed. After centrifugation, the supernatant fluid was deproteinized with basic lead acetate; the excess of lead was precipitated with H_2S and the H_2S removed by aeration. The solution was neutralized, filtered and concentrated *in vacuo*, at 40°, to 50 ml. The clear solution had $[\alpha]_D = +8·6°$, and was almost non-reducing. Incubation with invertase and treatment with hot 0·01 N-acid failed to liberate reducing sugar or to bring about any change in optical rotation. After total hydrolysis with hot N-acid, the solution reduced Bertrand (1 ml. solution gave 85·5 mg. Cu = 44·5 mg. 'glucose') and Auerbach-Bodländer (1 ml. solution required 5·1 ml. 0·1 N-$Na_2S_2O_3$ = 45·9 mg. aldose) reagents to the same extent. The observed rotation was $[\alpha]_D = 2·4°$; hence $[\alpha]_D = \frac{2·4 \times 100}{4·5} = +53·3°$.

The hydrolysate presumably contained only glucose. On this basis the amount of the parent oligosaccharide may be calculated as $45 \times 342/360 = 42·7$ mg. in 1 ml., and its specific rotation as

$$[\alpha]_D = \frac{8·6 \times 100}{42·7} = +201°,$$

in good agreement with the specific rotation ($[\alpha]_D = +197°$) of trehalose. The disaccharide present amounts to 7 % of the raw material.

In a further experiment, a second sample of raw material (14 g.) was treated according to the method of Harding (1924) for the preparation of trehalose from *Selaginella*. The substance was thrice extracted with 50–75 ml. boiling ethanol; the extract was then evaporated and the residue taken up in water. After elimination of the fermentable sugar with yeast, deproteinization, as previously described, and decolorization with charcoal, the solution was concentrated to a thin syrup and diluted with hot ethanol. The aqueous ethanolic solution was seeded with a few trehalose crystals. On slow concentration in a desiccator over P_2O_5 with repeated additions of ethanol, a crystalline deposit was formed. The substance was filtered off and washed with cold ethanol and ether. Yield 0·41 g., m.p. 96°. The hydrated form of trehalose melts at 97°. On drying at 78° over P_2O_5 *in vacuo*, 0·362 g. of the material lost 0·035 g. = 9·7 %. Calc. for $C_{12}H_{22}O_{11}.2H_2O$: H_2O, 9·5 %. A solution of the anhydrous sugar in water was non-reducing; $[\alpha]_D = +198·5$ ($c = 0·327$). The recorded value for trehalose is +197° (Schukoff, 1900).

It is already recorded that trehalose may be obtained from the so-called *Trehala* manna, described as an excretion of a larva or beetle (Meyer & Jacobsen, 1913), or as the product of the plant *Echinops persicus* on which the beetle lives (Wehmer, Thies & Hadders, 1932).

SUMMARY

1. A manna found in the North Iraqian Desert and said to be the excretion of a scale insect contains about 7 % of trehalose.

2. A sugar syrup prepared from this manna contains as much as 60 % trehalose.

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The Use of Bromine in the Estimation of Cellulose in Plant Materials

By J. G. SHRIKHANDE, *Tea Research Institute of Ceylon, Talawakelle*

(*Received* 22 *March* 1944)

A number of methods are available for the quantitative estimation of cellulose in plant materials and have been reviewed by Dorée (1933). Almost all of them are based upon the original procedure of Cross & Bevan (1903), who used chlorine gas for the removal of lignin as 'lignone chloride' followed by hot sodium sulphite extraction. Since then Jenkins (1930) and Norman & Jenkins (1933) have proposed the use of chlorine in solution as sodium hypochlorite to obviate the inconvenience of using gaseous chlorine. The defects in the original Jenkins (1930) method were subsequently discussed by Norman & Jenkins, who developed a modified method which eliminated them. Chlorination in this method is effected by acidification of dilute hypochlorite solution and, since the gas is liberated in contact with the material, treatment is effective and sometimes reduces the number of chlorinations necessary.

Although the use of chlorine in solution is advantageous it is difficult to obtain and store sodium hypochlorite in the tropics owing to its instability. In order to obviate a return to the use of gaseous chlorine, it was thought that sodium hypobromite, which is more easily prepared than the hypochlorite, might prove equally useful in cellulose determination, or that, alternatively, nascent bromine might well be liberated in contact with the material by acidifying a mixed solution containing bromide and bromate. After a few tests the method seemed to work satisfactorily enough to warrant the working out of a detailed analytical procedure based on the principles laid down in the chlorination processes. Although Dorée (1933) and Norman (1937), two of the more recent authors on cellulose chemistry, make no reference to the use of bromine in cellulose determination, Schwalbe (1938) has referred to the application of bromine for this purpose by Hugo Müller in 1877 in the form of hypobromous acid, and by Willstätter in 1913 as bromine water. Since the concentration of bromine available in these reagents for the bromination of cellulosic material is so small, the number of brominations has to be indefinitely increased. Consequently the process becomes long and laborious. The improved method described in this communication eliminates most of these defects.

The reactions involved are identical in kind with those for chlorine, either gaseous or in solution. Instead of the 'lignone chloride' a corresponding bromide is formed which under acid conditions possesses a yellow colour like the chloride; it produces a purple colour in cold sodium sulphite, although the intensity of colour is lower than with the chloride, and this colour disappears on heating.

Nascent bromine is liberated according to the following equation:

$$5KBr + KBrO_3 + 3H_2SO_4 = 3K_2SO_4 + 6Br + 3H_2O.$$

A similar reaction with chloride and chlorate does not liberate chlorine in the cold but some is evolved on heating. Since 0·75 g. chlorine is used by Norman & Jenkins (1933) at each stage under alkaline conditions, amounts of bromide and bromate can be calculated from the above equation to generate equivalent amounts of bromine in presence of H_2SO_4. Nearly 1 g. KBr and 0·25 g. $KBrO_3$ are needed for each stage in the new method. For bromination under alkaline conditions an equivalent amount of NaOH can be calculated from the following equation and added to the acidified mixture:

$$2NaOH + Br_2 = NaOBr + NaBr + H_2O.$$

The use of a bromide-bromate mixture as a source of bromine eliminates the handling of any elementary halogen, and the solution is stable at all temperatures and is easy to prepare. The mixture also supplies bromine easily under either acid or alkaline conditions. For acid brominations bromide-bromate solution is added, followed by a molecular equivalent of H_2SO_4. For alkaline bromination the equivalent amount of NaOH is added some time after the addition of acid; this delay is necessary since, as judged by the colour and smell of the liberated bromine, it takes some time for bromine liberation to develop.

EXPERIMENTAL

Solutions required: (1) *Bromide-bromate solution.* To a 10% solution of KBr add 2·5 g. of $KBrO_3$/100 ml. of solution; warm gently to dissolve the bromate. (2) 10% w/v H_2SO_4. (3) 5% NaOH. (4) 6% Na_2SO_3.

Comparison of various procedures in cellulose estimation

1 g. of pulverized cane-reed passing through a 64-mesh sieve was treated according to the recommendations of Norman & Jenkins (1933), substituting NaOBr or a mixture of bromide-bromate for NaOCl. The mixture was allowed to stand for 15 min. instead of the 10 recommended by these authors, washed with water on a poplin filter and then transferred to a beaker, the volume being made up to 100 ml. with 3% Na_2SO_3. Several experiments were

conducted with different permutations and combinations of acid and alkaline brominations to obtain the cellulose content which was the nearest approach to the value obtained by the Norman & Jenkins hypochlorite method, bearing in mind the effect of such treatments on the cellulosan and apparent lignin content of the cellulose product. The results of some of these experiments are given in Table 1.

Table 1. *Comparison of results with various combinations of brominations under acid and alkaline conditions, with cane-reed* (Pennisetum *sp.*)

Results expressed in g./100 g. of original material.
Acid and alkaline treatments are indicated by A and N respectively.

Treatment	Cellulose product	Xylan in cellulose product	Lignin in cellulose product	True cellulose
(1) NaOCl in 5 N-alkali	42·52	11·25	1·16	30·11
(2) NaOBr in 5 N-alkali	42·60	11·22	1·50	29·92
(3) NaOBr in 8 N-alkali	30·94	8·84	1·56	29·54
(4) 6A-1N-1A*	42·20	10·08	1·20	30·90
(5) 2N-2A-½A-½N†	44·54	9·98	2·00	32·56
(6) 3N-2A-1N	47·46	12·30	2·24	32·92
(7) N-A-N-A-N-A-N	43·00	10·04	1·40	31·56
(8) 1N-5A	51·86	14·70	1·80	35·36
(9) 2A-2N-A-N	44·70	11·05	2·32	31·33

* I.e. six brominations in acid, followed by one in alkali and then one in acid.
† ½A-½N indicate that each treatment lasted for 7–8 min. with no intermediate sulphite boil.

It will be noted from Table 1 that NaOBr of the same alkaline strength as the NaOCl gave similar results in respect of every constituent. It should, however, be mentioned that, with the use of NaOBr, one more acid treatment was needed than with NaOCl, in order to obtain a product which gave negligible colour in cold sulphite. NaOBr of a higher alkaline concentration affected the cellulosan fraction adversely. With a bromide-bromate mixture, six acid treatments followed by one alkaline and one acid bromination gave results comparable with those obtained by the hypochlorite method. The appearance of the product after alkaline treatment is always lighter in colour than after an acid treatment. The alkaline product does not give a purple colour in cold sulphite.

Treatment (4) gave a result approximating most nearly to that from the NaOCl method. Bromination under these conditions was found to be harmless to pure cellulose. This was tested by brominating finely ground Whatman no. 1 filter paper. Controls with equal numbers of sulphite and water extractions were conducted simultaneously. It will be noted from Table 2 that the amounts of cellulose recovered after bromination and sulphite extractions were similar to those obtained from sulphite or water extractions.

Table 2. *Results of estimations on pure cellulose* (*filter paper*)

Treatment	Cellulose recovered from 1 g. of filter paper
6A-1N-1A*	0·974
N-A-N-A-N-A-N	0·975
Six boilings in Na_2SO_3 (20 min. each)	0·975
Six boilings in water (20 min. each)	0·979

* I.e. six brominations in acid, followed by one in alkali and then one in acid.

Standard method

Based on the above experiments, the following method has been evolved and made applicable to different kinds of materials. The material should be in a uniform state of division; 64–80 mesh seems quite suitable, but for tender green materials 64 mesh is preferable, since the cellulose product tends to gelatinize as bromination proceeds and the filtration is then extremely slow. 1–2 g. of the material, depending upon its age and nature after alcohol-benzene extraction, are stirred in 100 ml. of 3% Na_2SO_3, brought to the boil and filtered. The washed material is then transferred back to the beaker with 100 ml. of water, and 10 ml. of bromide-bromate mixture are added, followed by 5 ml. of 10% (w/v) H_2SO_4. The mixture is allowed to stand 15 min., with occasional stirring. The liquor is then sucked off on a cloth filter and the material washed with water. The washed material is retransferred to the container with 50 ml. of water and 50 ml. of 6% Na_2SO_3 are then added. The contents are brought to the boil and the beaker left in a boiling water-bath for 20 min. The process of acid-bromination is repeated till the purple colour due to 'lignone bromide' in cold sulphite is very faint. This may take five to six acid-brominations, depending upon the age and nature of the material. The cellulose product, which is by this time much lighter in appearance, is then suspended in 100 ml. of water, to which are added 10 ml. of bromide-bromate mixture followed by 10 ml. of H_2SO_4. After the development of the reaction, 5% NaOH (about 10 ml.) is added till the liquor in the beaker is colourless; 15 min. are allowed for alkaline bromination. The material, after being washed, presents a bleached appearance and it is extracted in 100 ml. of 3% Na_2SO_3 as before. In the absence of a simple and reliable test for the removal of lignin, one more acid treatment is given to ensure that the material is completely delignified, as indicated by the absence of purple coloration in cold sulphite extract of the brominated material. Sodium sulphite is finally removed by boiling the final cellulose product in about 250 ml. of water for 2 min. After thorough washing on the filter the product is transferred by a spatula to a weighed Gooch crucible with a cloth disk. This facilitates rapid filtration in the crucible. The crucible is dried at 100° and weighed, and the product is powdered for furfural and lignin determination.

Though the method is somewhat longer than the hypochlorite method, when the reagent for the latter is not easily available, bromide-bromate solution for halogenation appears to be satisfactory. Moreover, for cellulose determination in tender green materials which are not highly lignified, bromine has an advantage over chlorine in that, owing to its lesser reactivity, there is less chance of the tissue being damaged by over-halogenation.

Table 3 shows results obtained for certain materials by the new method, compared with the corresponding figures obtained by the Norman & Jenkins (1933) procedure. From the evidence it is apparent that the method is capable of giving results comparable with those of the hypochlorite determinations. The method can also be applied to large-scale preparations in the same manner as the hypochlorite process.

Table 3. *Comparison of results obtained by the hypochlorite method and our new method*

Results expressed in g./100 g. original material

Material	Norman & Jenkins method: Cellulose product	Xylan in cellulose product	Lignin in cellulose product	True cellulose
Tephrosia vogelii	25·00	1·28	2·32	21·40
Grass cuttings	34·40	6·62	2·08	25·70
Tea leaf	18·34	2·37	2·38	13·59
Paddy straw	41·76	7·51	3·00	31·25

Material	Bromination method: Cellulose product	Xylan in cellulose product	Lignin in cellulose product	True cellulose
Tephrosia vogelii	23·20	0·98	1·80	20·42
Grass cuttings	33·00	6·00	2·40	24·60
Tea leaf	19·14	2·23	3·02	13·89
Paddy straw	40·66	6·95	2·72	30·99

SUMMARY

1. A new method for the determination of cellulose in plant materials, based on the use of bromine as a halogenating agent for lignin, is described. Bromine is liberated in the nascent state in contact with the material by acidifying a mixed solution of bromide and bromate. Bromination under alkaline conditions can also be effected by adding an equivalent amount of NaOH to the acidified mixture. The reactions involved in the process are similar to those in the hypochlorite methods. Results obtained by the use of either NaOBr or a mixture of bromide and bromate are comparable to those given by the NaOCl method.

2. The method is distinctly advantageous in the tropics, where it is difficult to obtain and store NaOCl. One or two treatments (depending upon the nature and age of material) in excess of those needed in the NaOCl method are required, since bromine is less reactive than chlorine. This lesser reactivity is an advantage in the case of tender green plant tissues, which are liable to damage by over-chlorination.

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Palm Oil Carotenoids

3. EXAMINATION OF THE LIPOID PIGMENTS PRESENT IN 'MALAY' AND 'BISSAO' PALM OILS

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The new type of palm grown on plantations in the Malay Peninsula was especially selected to furnish an oil of much paler tint than the palm oil produced commercially in West Africa. It was therefore of interest to compare the carotenoids present in Malay oil with those which occur in a deep red palm oil such as that obtained from the Portuguese island of Bissao.

Samples of both types of oil were examined on the lines described in our first paper (Hunter & Scott, 1941). Chromatographic analysis of the petrol-soluble unsaponifiable matter of 'Malay' palm oil furnished crystalline specimens of α- and β-carotene. Evidence was also obtained of the presence of lycopene, γ-carotene, and the carotenoid which is adsorbed at a position intermediate between γ- and β-carotene on the chromatogram (Hunter & Scott, 1941). The ether-soluble unsaponifiable matter furnished somewhat ill-defined xanthophylls having absorption spectra similar to those of the *neo*luteins (Zechmeister & Tuzson, 1938).

The amount of 'Bissao' palm oil at our disposal was very limited, but an examination of this indicated the presence of lycopene, *neo*lycopene and γ-carotene in addition to the α- and β-carotenes which are mainly responsible for the deep red colour.

Both palm oils therefore show a close similarity to those previously examined with regard to the carotenoids present (Hunter & Scott, 1941; Hunter, Scott & Edisbury, 1942).

EXPERIMENTAL

Malay palm oil

The sample of 'Malay' palm oil used showed an extinction coefficient of 0·78 at 458 mμ in chloroform.

The oil (1500 g.) was saponified in two batches of 750 g. as described for 'Sherbro' palm oil (Hunter & Scott, 1941). The soap solution was diluted with ethanol (3 l.), made up to 10 l. with water, and thrice extracted with light petroleum, and the united extracts were washed, dried with Na_2SO_4, and evaporated under reduced pressure in an atmosphere of hydrogen, with the usual precautions. The extracted soap solution was diluted with water, thrice extracted with methylated ether, and the combined extracts washed, dried, and evaporated.

The petrol-soluble unsaponifiable matter (2·77 g.) was dissolved in a 20% solution of benzene in light petroleum, chromatographed on 'Birlec' (Birmingham Electric Furnaces Ltd.) alumina, and the chromatogram developed with 30% benzene in light petroleum. On elution, the carotene zone furnished 0·516 g. of semi-solid mixture of α- and β-isomers which was rechromatographed on 'Mayfair' (Savory and Moore, Ltd.) alumina. The concentrates of α- and β-carotene thus obtained were again rechromatographed.

α-Carotene separated from acetone in glistening red-brown crystals, m.p. 184–185°, which showed absorption maxima at 511, 479 and 449 mμ in carbon disulphide, at 490, 460 and 431 mμ in benzene, and at 490, 458 and 429 mμ in chloroform. This specimen had $E^{1\%}_{1\,cm.} = 2250$ at 460 mμ in chloroform and showed $[\alpha]_D = +620°$ in benzene. β-Carotene had m.p. 182–183° after several recrystallizations, showed absorption maxima at 516 and 486 mμ in carbon disulphide, and had $E^{1\%}_{1\,cm.} = 2200$ at the highest absorption band in chloroform.

The material eluted from the upper portions of the original chromatogram was rechromatographed on 'Birlec' alumina and the chromatogram developed with 30% benzene in light petroleum. The following series of zones was obtained in order of decreasing adsorption: several very narrow dark orange-red zones treated as zone 1, orange (narrow, zone 2), deep orange (zone 3), purple-brown (broad, zone 4), orange (broad, zone 5), and pale orange (very broad, zone 6).

Zones 1 *and* 2. The substances eluted from zones 1 and 2 showed very vague absorption spectra and were therefore combined and rechromatographed, and the chromatogram developed with 50% benzene in light petroleum and finally with benzene alone. An intermediate orange zone was obtained which on elution, and further chromatography, gave a carotenoid which showed absorption maxima at 506 and 478 mμ in carbon disulphide and at 486 and 456 mμ in chloroform.

Zone 3 furnished lycopene which was rechromatographed on 'Birlec' alumina. The material eluted from the main lower orange zone showed absorption maxima at 543, 503, and 474 mμ in carbon disulphide.

Zone 4 gave an eluate which appeared to be a mixture of lycopene and γ-carotene.

Zone 5 furnished a carotenoid having the spectroscopic characteristics of γ-carotene. After being rechromatographed this showed absorption maxima at 528, 494, and 463 mμ in carbon disulphide and a band at 594 mμ in the antimony trichloride reaction.

Zone 6 furnished a carotenoid which showed absorption maxima at 485 and 454 mμ and an inflexion at 425 mμ in carbon disulphide. The zone below this (*zone* 7) contained similar material.

The ether-soluble unsaponifiable matter (2·66 g.) in a 25% solution of benzene in light petroleum was chromatographed on 'Birlec' alumina. The chromatogram developed by 40% benzene in light petroleum formed a series of zones: (1) light orange, (2) cream, (3) light pink, and (4) dark cream, in order of decreasing adsorption.

Zone 1 furnished an orange-coloured slightly gummy material which showed absorption bands at 503 and 473 mμ in carbon disulphide but which could not be satisfactorily purified by further chromatography. The zone below this had little colour and was therefore neglected.

Zone 3 furnished yellow gummy material which showed a maximum at 479 mμ with inflexions at 505 and 451 mμ in carbon disulphide.

Zone 4. The material from zone 4 was dissolved in a 5% solution of benzene in light petroleum and rechromatographed on alumina which had been 'weakened' by exposure to air. The narrow upper orange-coloured zone of the chromatogram and the yellow zone below this gave substances with closely similar absorption spectra (maxima at 503 and 470 mμ with an inflexion at 447 mμ and at 502 and 471 mμ with an inflexion at 446 mμ in carbon disulphide respectively) which were combined, dissolved in ethanol and the sterols removed with digitonin. The carotenoid thus obtained showed absorption maxima at 503, 472, and 443 mμ in carbon disulphide and a band at 584 mμ in the antimony trichloride reaction.

Bissao palm oil

The sample of oil used showed an extinction coefficient of 5·3 at 458 mμ in chloroform.

The oil (510 g.) was saponified, and the petrol-soluble unsaponifiable matter in a 20% solution of benzene in light petroleum was chromatographed on 'Birlec' alumina. The chromatogram formed the following series of zones, in order of decreasing adsorption: (1) narrow brown, (2) narrow pink, (3) narrow pink, (4) light red, (5) red-brown, (6) orange, (7) red-brown, (8) orange, (9) yellow, and (10) broad orange red (carotene zone).

The material eluted from *zone* 1 showed a vague band at 485 mμ and that from *zone* 2 absorption maxima at 485 and 450 mμ in carbon disulphide. *Zones* 3 and 4 contained lycopene which after recrystallization from acetone-methanol had m.p. 158° and showed maxima at 547, 507, and 478 mμ in carbon disulphide.

Zone 5 furnished a small amount of material showing absorption maxima at 535, 499, and 465 mμ in carbon disulphide. Zechmeister & Tuzson (1938) record 536, 498, and 466 mμ for the absorption maxima of *neo*lycopene in carbon disulphide.

Zone 6 also appeared to contain *neo*lycopene.

Zone 7 furnished a carotenoid having the spectroscopic characteristics of γ-carotene (maxima at 530, 496, and 465 mμ in carbon disulphide after further chromatography).

Zones 8 and 9 furnished carotenoids showing absorption maxima at 485 and 465 mμ and at 455 and 429 mμ in carbon disulphide respectively.

The carotene zone furnished a mixture of α- and β-carotenes whose dextrorotation in benzene indicated the presence of 28% of the former isomer (see Hunter & Scott, 1944).

The ether-soluble unsaponifiable matter was dissolved in light petroleum and the solution was extracted ten times with 90% methanol. The residue obtained from the united extracts was chromatographed on 'Birlec' alumina and thereafter on calcium oxide. The broad orange-yellow upper zone furnished a carotenoid which showed absorption maxima at 500 and 473–474 mμ in carbon disulphide.

SUMMARY

1. Examination of the unsaponifiable matter of a 'Malay' palm oil showed the presence of α-, β-, and γ-carotenes, lycopene, the carotenoid intermediate between γ- and β-carotene on the chromatogram, and xanthophylls having absorption spectra similar to those of lutein and *neo*lutein.

2. Examination of a sample of 'Bissao' palm oil showed the presence of lycopene, *neo*lycopene, and γ-carotene in addition to the α- and β-carotenes which are mainly responsible for the deep red colour of this oil.

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Palm Oil Carotenoids

4. THE PROPORTION OF α- TO β-CAROTENE IN UNRIPE AND RIPE PALM OILS AND ATTEMPTED INTERCONVERSION OF α- AND β-CAROTENES

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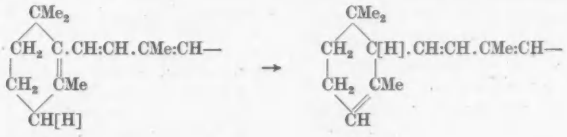
It was suggested in our first paper (Hunter & Scott, 1941) that the α-carotene present in palm oil may arise from asymmetric isomerization of β-carotene by way of a three-carbon prototropic change of the type analogous to the racemization of mobile azomethine derivatives (Hsu, Ingold & Wilson, 1935). This isomerization is depicted by the partial formulae (I) and (II) in which [H] represents the mobile hydrogen atom:



(I) (II)

On this basis, the proportion of α-isomer might be expected to vary with the degree of ripeness of the palm fruit. We have now examined a number of samples of oil obtained from unripe and ripe bunches of fruit collected from the same trees of a West African palm plantation. The amounts of the samples available were necessarily small and permitted only a single determination of the proportion of α- to β-carotene present in them. A qualitative examination was also made of the other carotenoids present in the petrol-soluble and ether-soluble unsaponifiable matter obtained from the samples.

In the first investigation, an examination was made of three pairs of samples of palm oil obtained from unripe and ripe bunches of fruit, two pairs being from fruits of the *Afia Oku* variety and one pair from the *Lisombe* variety. The results, which are summarized in Table 1, indicate that there is little difference between the proportion of α- to β-carotene in oil obtained from unripe and ripe fruits from the same palm, although there is quite a wide variation from tree to tree.

It appeared possible that the unripe fruit which had been pressed to obtain these samples might have been too 'ripe' for the purpose in view. A second investigation was therefore carried out in which particularly immature fruit, furnishing less than 10% of oil, was used to provide the samples of unripe oil. The results are summarized in Table 2.

Table 1. *Proportion of α- to β-carotene in oil from ripe and unripe fruits*

Sample	Carotene (α+β) (%)	α-Carotene (% of total)	Lycopene	γ-Carotene	Lutein (or xanthophyll with similar absorption)	Oil content of fruit (%)	Oil content of pericarp (%)	Free fatty acid (%)
Unripe *Afia Oku* (*a*)	0·0508	35	—	Present	Present	28·5	37·3	1·3
Ripe *Afia Oku* (*a*)	0·058	35	Present	Present	Present	34·3	43·2	1·6
Unripe *Afia Oku* (*b*)	0·0685	25	Not detected	Not detected	—	30·8	37·9	0·75
Over-ripe *Afia Oku* (*b*)	0·0785	27	Not detected	Not detected	Present	45·9	55·8	7·4
Unripe *Lisombe*	0·0495	43	Present	Present	Present	23·5	27·5	0·65
Ripe *Lisombe*	0·0825	39	Present	Present	Present	41·6	48·5	0·88

Table 2. *Proportion of α- to β-carotene in oil from very immature and ripe fruits*

Sample	Carotene (α+β) (%)	α-Carotene (% of total)	Lycopene	γ-Carotene	Lutein (or xanthophyll with similar absorption)	Oil content of fruit (%)	Oil content of pericarp (%)	Free fatty acid (%)
Unripe *Afia Oku*	0·0473	43	Not detected	Not detected	Present	7·3	15·8	2·0
Ripe *Afia Oku*	0·0688	47	Not detected	Present	Present	17·8	31·8	0·7
Unripe *Lisombe*	0·0165	42	Not detected	Not detected	Present	7·7	22·6	—
Ripe *Lisombe*	0·0627	40	Not detected	Not detected	Present	24·1	63·9	2·66

As in the case of the first investigation, the proportion of α- to β-carotene in the oils obtained from ripe fruit did not differ substantially from that in the corresponding oil from unripe fruit.

The prototropic triad system involved in the hypothetical isomerization of β-carotene to α-carotene is of the true three-carbon type such as is present in the methoxydiphenylpropenes (III) and (IV) which were first thought to be static (Ingold & Piggott, 1922) and later found to undergo interconversion under the catalytic influence of ethoxyl ions (Ingold & Shoppee, 1929):

(III) (IV)

An attempt to convert β-carotene into α-carotene by treatment with sodium *iso*propoxide in *iso*propanol, which is a stronger catalyst than sodium ethoxide for prototropic change (Kon & Linstead, 1929), proved unsuccessful. A similar attempt to convert α-carotene into the β-isomer, in which the entry of the double bond into the conjugated system might be expected to lower the activation energy, gave a similarly negative result.

EXPERIMENTAL

First investigation. The oil content is regarded as a criterion of the degree of ripeness of palm fruit, and the figures for this (which were determined for us in West Africa) are shown in Table 1 along with our own determinations of the free fatty acid content of the samples.

The method of examination used may be illustrated by the case of the first sample of unripe *Afia Oku* (*a*).

The oil (350 g.) was saponified with KOH (105 g.) in water (175 ml.), ethanol (550 ml.), and light petroleum (350 ml.), the temperature being kept below 50°. The mixture was heated to boiling, allowed to cool, kept overnight at laboratory temperature and thereafter heated under reflux for an hour. The solution was cooled, diluted with ethanol (2 l.), then with water to produce a final dilution of 5 l. and twice extracted with light petroleum. The united extracts were washed, dried with $MgSO_4$, and evaporated in an all-glass apparatus with the usual precautions. The residual soap solution was diluted with water to 7 l. and extracted with ether.

The petrol-soluble unsaponifiable matter was chromatographed on 'Birlec' alumina under slight CO_2 pressure, a 20% solution of benzene in light petroleum being used for development of the chromatogram. This formed a series of seven zones: (1) olive green, (2) narrow brown, (3) narrow dark red, (4) red, (5) yellow, (6) broad orange red typical carotene, (7) pale yellow, in order of decreasing adsorption. The mixture of α- and β-carotenes obtained from zone (6) was dissolved in benzene and the solution was made up to 50 ml. and used for the optical measurements.

1 ml. of the solution was diluted with chloroform to 50 ml. and this was used for spectroscopic measurements. The most intense maximum in the carotene absorption spectrum in chloroform occurs near 460 mμ (458 for α-carotene and 463 mμ for β-carotene) and this point was therefore used for the intensity measurements. The mean of ten observations gave a $\log I_0/\log I$ value of 1·661, corresponding to a concentration of 0·356% of carotene in the original solution.

1 ml. of the original benzene solution was then diluted with benzene to 20 ml. and this was used for the polarimetric estimation of α-carotene. The concentration must lie between 0·016 g./100 ml. and 0·022 g./100 ml., so that the observed rotation shall be as high as possible, and at the same time the light transmitted by the solution shall be sufficient for making the determination. This gave a value of

$[\alpha]_D = +225°$, corresponding to the presence of 35% of α-isomer on the basis of α-carotene having $[\alpha]_D = +640°$ (Hunter & Scott, 1941).

The upper zones of the chromatogram were combined and eluted, and the residue obtained by evaporation was dissolved in light petroleum and rechromatographed on magnesium sulphate (100–120 mesh, previously heated at 250° for ½ hr.) when a chromatogram similar to the original, but more dispersed, was obtained.

Zones 1 and 2 gave substances having indefinite spectroscopic characteristics. The material eluted from zone 3 showed an inflexion at 505 mμ in carbon disulphide and an absorption band at 590 mμ in the antimony trichloride reaction. The ultra-violet absorption spectrum in *cyclo*hexane showed a band at 295 mμ, presumably attributable to tocopherol (cf. Emerson, Emerson, Ali Mohammad & Evans, 1937). The material eluted from zone 4 showed absorption maxima at 502 and 474 mμ in carbon disulphide. The ultra-violet absorption spectrum of this also showed a band at 295 mμ in *cyclo*hexane. Zone 5 contained γ-carotene or neo-γ-carotene (maxima at 523 and 489 mμ in carbon disulphide). The material washed out from zone 7 showed general absorption in the ultra-violet in *cyclo*hexane with slight maxima at 335 and 405 mμ.

The ether-soluble unsaponifiable matter was chromatographed on magnesium sulphate, when an upper yellow zone and a lower orange-yellow zone were obtained. The former of these furnished lutein, or a mixture of this with *neo*lutein (maxima at 504 and 474 mμ in carbon disulphide and a transient band at 620 mμ in the antimony trichloride reaction; cf. Hunter, Scott & Edisbury, 1942). The substance obtained from the lower zone showed maxima at 503 and 474 mμ in carbon disulphide, and gave an olive green colour changing to blue-green in the antimony trichloride reaction.

The oil from ripe *Afia Oku* corresponding to the foregoing (200 g.) was saponified as in the previous case. The petrol-soluble unsaponifiable matter, on being rechromatographed on magnesium sulphate, furnished a zone containing lycopene (maxima at 545 and 506 mμ and an inflexion at 475 mμ in carbon disulphide) in addition to γ-carotene (maxima at 527 and 493 mμ and an inflexion at 455 mμ in carbon disulphide).

Second investigation. The oil contents of the fruit used in preparing the samples and the free fatty acid contents are shown in Table 2. With the exception of unripe *Lisombe* of which only 60 g. were available, 200 g. of oil were saponified in each case.

Attempted interconversion of α- and β-carotenes. (1) Sodium (2·3 g.) was dissolved in *iso*propanol (50 ml. previously treated with a small amount of sodium and redistilled) to give a 2 N solution of sodium *iso*propoxide which was heated under reflux during the addition of α-carotene (10 mg., m.p. 186°) in benzene (5 ml.). Heating was continued for 5 hr. and the mixture was kept overnight at laboratory temperature. Water (100 ml.) was added together with light petroleum and a small quantity of benzene, and the hydrocarbon layer was washed free from alkali and evaporated under reduced pressure in CO_2 atmosphere. The residue was dissolved in benzene (5 ml.) and the solution diluted with light petroleum and chromatographed on 'Mayfair' (Savory and Moore, Ltd.) alumina. The chromatogram formed an upper orange zone and a lower pale yellow zone, containing unchanged α-carotene (maxima at 508 and 477 mμ in carbon disulphide) and neo-α-carotene (maxima at 498 and 468 mμ; cf. Gillam, El Ridi & Kon, 1937) respectively.

(2) A similar experiment was carried out in which β-carotene (m.p. 181–182°) in benzene was added to 2 N-sodium *iso*propoxide in *iso*propanol and the mixture was heated under reflux for 5 hr. Chromatographic adsorption of the product on 'Mayfair' alumina gave an upper orange-red zone and a lower orange-yellow zone, containing unchanged β-carotene (maxima at 516 and 485 mμ in carbon disulphide) and ψ-α-carotene (maxima at 505 and 478 mμ in carbon disulphide and a band at 590 mμ in the antimony trichloride reaction which faded with development of maxima at 660 and 590 mμ) respectively.

SUMMARY

1. A number of samples of oil was prepared from specially selected bunches of unripe and ripe palm fruit. While the total carotene content varied considerably, the proportion of α- to β-carotene in oils obtained from unripe and ripe fruit of the same tree remained constant. The proportion of isomers varied, however, from tree to tree.

2. A qualitative investigation was also made with regard to the presence of lycopene, γ-carotene and xanthophylls of the lutein type present in the samples.

3. Attempts to effect the interconversion of α- and β-carotene by heating with sodium *iso*propoxide in *iso*propanol proved unsuccessful.

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The Chemistry of Wheat Germ with Particular Reference to the Scutellum

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In an earlier paper, attention was directed to the surprising concentration of aneurin in the scutellum of wheat (Hinton, 1942). A preliminary figure of 40 i.u./g. was reported as compared with 4 i.u./g. for the next known highest portion of the wheat grain—the embryonic portion of the germ. Ward has also published results which confirm these figures (Ward, 1943).

The present paper reports (*a*) the results of further work on aneurin distribution in eleven wheat types and four other cereal grains, (*b*) the results so far obtained in a systematic investigation of the chemistry of the scutellum and embryo of wheat which is being undertaken in these laboratories.

METHODS

Aneurin. The determinations of aneurin were made by the thiochrome method (Nicholls, Booth, Kent-Jones, Amos & Ward, 1942), those on the embryo and scutellum by a micro modification (Hinton, 1943). The embryo and scutellum were dissected from well-formed, whole, dry, individual grains of which about 20 were required to obtain a sufficient sample (5–10 mg.), and the aneurin was extracted by standing overnight at room temperature in 0·2 N-HCl.

Other constituents. The rest of the analytical figures were obtained by normal methods applied on a micro-chemical scale, with 10–20 mg. of material dissected as described above, the figures being expressed on an air-dry basis.

N was estimated by Kjeldahl's method; P by Allen's (1940) method; total P, material digested in HNO_3 and H_2SO_4; lipoid P, material digested in HNO_3 and $HClO_4$; phytate P, material extracted by 0·5 N-HCl and doubly precipitated with Ca and Mg (Leva & Rapoport, 1941);* phosphatase, details of the method of estimation will shortly be published by Dr R. G. Booth; lipoid, extracted by petrol ether in a reflux type of extractor. The lipoid material was dried in a vacuum desiccator at room temperature; no further loss of weight was experienced in subsequent heating at 100° for 1 hr.

RESULTS

(*a*) *Aneurin distribution in wheats and other cereals*

The samples of grain were taken from ordinary commercial parcels of unknown history and, with three exceptions, of unknown variety. In some cases figures represent duplicate or triplicate dissections of the embryo and scutellum, in others single dissections (Table 1). From the former it appears that the aneurin figures as a whole may be subject to a sampling variation of about 50 % in the case of the embryo and about 10 % in the scutellum and whole grain. The higher variation in the embryo is probably due to the difficulty of making a perfect dissection free from scutellum rather than to greater variation in its true aneurin content.

The average proportions of the total aneurin contributed by the scutellum (less than 2 % of the grain weight) and the embryo are 59 and 3·1 % respectively. It is interesting to note the high concentration in the scutellum of the hard Durum and Egyptian (which may well be a Durum variety), and the lower concentration in the soft English varieties. The Russian, also high, was a hard red type, very similar in appearance to the Garnet. The figures are, however, too few to be conclusive.

In none of the other cereals examined was the concentration of aneurin in the scutellum as high as in the wheats; in maize, however, the scutellum contributed an even greater proportion of the total aneurin as a result of its large size.

Aneurin content of plumule and radicle portions of wheat. The embryo of Squarehead's Master was divided into two, the radicle and plumule portions. The aneurin contents of the two were:

Plumule (44 % of the embryo)	3·1 i.u./g.
Radicle (56 % of the embryo)	2·4 i.u./g.

Whilst carrying out these estimations it was noted that the water-soluble indicator dye present in wheat germ was confined mainly to the radicle portion. A rapid method for estimating the amount of germ present in cereal products has been put forward, based on the presence of this dye, a water extract being made alkaline and the bright yellow colour produced measured in a colorimeter. This procedure will, however, give reliable results only if the radicle portion is a fixed proportion of the whole germ and this proportion is faithfully represented in the germy material under test.

* Phytate P and total P determinations were carried out by Mr W. J. S. Pringle and it should be mentioned that he has evidence of complication, as yet unsolved, in the determination of phytin in wheat grain. The figures may therefore be amended as a result of further work.

Table 1. *Aneurin content of scutellum and embryo and their proportion of the grain weight of various wheats and other cereals*

Grains	Average weight of grain (g.)	Aneurin content (i.u./g.) of			Weight (g./100 g. of grain) of		Total aneurin content (% of total in grain) of	
		Grain	Embryo	Scutellum	Embryo	Scutellum	Embryo	Scutellum
				(*a*) Wheats				
Red Standard	0·055	1·1	1·4	41	1·15	1·74	1·5	65
Squarehead's Master	0·046	1·3	2·7	54	0·97	1·47	2·0	61
White English	0·045	1·3	2·0	53	0·96	1·38	1·5	56
Russian	0·039	1·4	3·1	67	1·03	1·31	2·3	63
No. 1 Garnet	0·034	1·5	4·3	55	1·53	1·78	4·4	65
American Hard Dark Winter	0·026	1·5	7·3	60	1·37	1·37	6·7	55
Egyptian	0·053	1·6	8·1	75	0·92	1·40	4·6	65
No. 1 Manitoba	0·035	1·6	3·1	56	1·30	1·64	2·5	57
No. 4 Manitoba	0·034	1·6	3·9	56	1·38	1·56	3·3	54
Australian	0·048	1·8	4·4	61	1·20	1·54	2·9	52
Durum	0·041	2·0	4·2	70	1·36	1·68	2·8	59
			Average	59	1·20	1·54	3·1	59
				(*b*) Other cereals				
Rye	0·032	0·8	2·3	38	1·80	1·73	5·2	82
Barley (husk removed)	0·052	1·1	5·0	35	1·85	1·53	8·4	49
Maize	0·330	1·2	8·7	14	1·15	7·25	8·35	85
Oats (husk removed)	0·038	1·7	4·8	22	1·60	2·13	4·5	28

Aneurin content of germinating wheat. The changes in the aneurin content of scutellum during germination have also been followed (Table 2).

Table 2. *Change in aneurin content of scutellum of wheat during germination*

Days of germination	Condition of seed	Aneurin in scutellum (i.u./g. dry wt.)
1	—	53
2	First root just visible	50
3	First root 1 cm. long	45
5	First root 3–5 cm. long	25

There is obviously an appreciable loss of aneurin from the scutellum when germination is well advanced. Unfortunately, because of the production of substances which seriously interfere with the thiochrome determination, it has not yet been possible to determine the corresponding aneurin values for the growing embryo and the endosperm.

Effect of soaking wheat in water. A sample of Squarehead's Master was soaked in water for different periods, dried, the scutellum dissected and assayed for aneurin, with the results given in Table 3.

Table 3. *Aneurin content of scutellum of wheat after the latter has been soaked in water*

	Aneurin (i.u./g.)
Control wheat	54
After 1 hr. soaking	53
After 3 hr. soaking	47
After 6 hr. soaking	54
After 12 hr. soaking	54

There is no evidence from these figures of a movement of aneurin from the scutellum. This observation is of some theoretical interest, since water enters the wheat grain most readily at the germ end, moving past the scutellum in its path and, further, aneurin is readily extracted from whole commercial germ by the dilute acid (0·2 N-HCl) used in preparing the extracts for estimation. The probable explanation for the absence of any transfer of aneurin is that the vitamin is extracted by water only when the cells of the scutellum have been made permeable by the action of acid, chloroform or some similar agent. This is borne out by the figures given in Table 4.

Table 4. *Aneurin extracted from scutellum of wheat under different conditions*

Scutellum:	Aneurin extracted (i.u./g.)
Powdered and extracted by HCl	52
Powdered and extracted by distilled water	31
Powdered and extracted by $CHCl_3$ water	51
Large pieces extracted by HCl	62
Large pieces extracted by distilled water	22
Large pieces extracted by $CHCl_3$ water	48

Tests showed that the low figures for the distilled water extracts were not due to decomposition of the vitamin, since solutions of aneurin remained stable in distilled water over the period of the experiment. The high figure of 62 i.u./g. for the large pieces of scutellum is in agreement with Ward's figures and suggests that the epithelium half of the

scutellum contains less aneurin than the remainder, since these pieces were cut out, leaving most of the epithelium behind. On another occasion a figure of 67 i.u. was obtained on a similar sample.

(*b*) *Other chemical characteristics of scutellum and embryo of wheat*

The figures quoted in Table 5 were obtained throughout on a sample of Squarehead's Master.

Table 5. *Chemical characteristics of scutellum and embryo of wheat*

	Total N (%)	Protein N × 5·27 (%)	Phosphorus				Phytic acid equiv. (%)	Phosphatase (King-Armstrong units)
			Total (%)	Phytate (%)	Non-phytate (%)	Phytate (as % total)		
Scutellum	5·04	28·7	1·90	1·311	0·586	69·1	4·65	58·0
Embryo	5·86	33·4	1·16	0·397	0·760	34·3	1·41	36·0* 56·0†

* Plumule portion of embryo. † Radicle portion of embryo.

The high protein figure in the scutellum, very little lower than that of the embryo, is not surprising since they are both dense tissues, formed mainly of unvacuolated cells. The higher phosphorus content, 64 % higher than the embryo, is more than accounted for by its greater content of phytate phosphorus.

The author's colleague, Dr R. G. Booth, has carried out determinations of the monophosphoesterase content of scutellum and embryo in dormant wheat on materials provided by the author. The results, incorporated in Table 5, are in general agreement with the findings of Pett (1935) that the scutellum has a greater enzyme content of dipeptidase, protease and lipase than the embryo.

Some constants of the petrol-ether extractable lipoid have been determined (Table 6).

Table 6. *Petrol-ether extractable lipoid of scutellum and embryo*

	Fat lipoid content (%)	Properties of lipoid		
		Acid no.	Iodine no. (Wijs)	P content (%)
Scutellum	30·3	5·3	116	0·02
Embryo	15·4	3·7	133	0·03

The petrol ether-extractable lipoid from the scutellum is double that from the embryo and is unusually high for a wheat product. The difference in acid number is probably not significant, with regard to the difficulties of measuring it, although in view of the fact that Pett reported approximately four times more lipase in the scutellum of dormant wheat grain than in the embryo, the oil from the former would be expected to have a higher acid number. The lower iodine number of the scutellum lipoid suggests that it is of different composition and it was thought that it might contain a larger proportion of phospholipins which would depress the iodine number. The phosphorus content of the oil from the scutellum, however, is lower than that from the embryo, so this cannot be the explanation. Incidentally, the phosphorus figures are lower than those previously reported for germ oil (see, e.g., Sullivan & Near, 1933), for ethyl ether extraction). The method of estimating the phosphorus was checked on a larger sample of germ oil by a parallel determination in which the oil was ignited with $Mg(NO_3)_2$ and the P estimated by the Embden-Fetter strychno-molybdate precipitation method. Good agreement between the two methods was obtained.

DISCUSSION

The results presented in this paper extend those previously reported (Hinton, 1942) and show that, in diverse types of cereal grains, the aneurin is concentrated to a greater or lesser extent into the scutellum, this being particularly true of the main commercial types of wheat. This finding may be of importance in the production of bread-making flour of high aneurin content. Thus in a 100 % wheatmeal, using the average figures shown, it can be calculated that the scutellum would account for 0·88 i.u. B_1/g. of flour. If the extraction is decreased, this contribution will increase and in the case of an 85 % extraction flour containing the whole of the scutellum, the latter would contribute 1·03 i.u. aneurin/g. of flour.

The aneurin content of the scutellum of the wheats examined varies from 41 to 75 i.u./g. At the same time, the proportion of the total aneurin of wheats found in the scutellum does not vary much from the average of 59 %, so that the importance of the scutellum is equally great in each case.

The problem of the milling of a high aneurin flour is simply that of the production of a flour containing the maximum amount of scutellum. However, the nature of the practical milling problem thus involved can be gauged by the fact that a variation of 10 % in the scutellum milled into the flour, that is, only 0·15 % of the grist, would produce a variation in aneurin content of the order of 0·1 i.u./g.

Of practical importance is the fact that no loss of aneurin from the scutellum as a result of soaking wheat in water could be detected. Doubt has existed whether the moistening of wheat grain, carried out to adjust suitably the moisture content in preparation for milling, might not produce a redistribution of the aneurin so that the endosperm might gain in the vitamin from the scutellum, which would consequently be of less importance. Such does not seem to be the case; the scutellum will be as important in moistened wheat as in dry. It follows likewise that adjustment of water content as a preparation for milling can be undertaken freely without affecting the validity of any technical development based on the high vitamin content of the scutellum.

A further discussion of the practical aspect of the subject, which should be read in the light of the extended range of figures given in the present paper, has been given by Moran (1942).

There is as yet no indication of the metabolic significance of this concentration of aneurin; no clear conclusions can be drawn from the figures obtained on germinating wheat. There was very little change in aneurin content of the scutellum at 3 days' germination, at which time the dry weight of the embryo had increased three times and erosion of the endosperm was well marked. At 5 days' germination, at which time the aneurin had fallen by half, the dry weight of the embryo had increased nine times and erosion of the endosperm was well advanced. At this time, the scutellum showed signs of physical change, being dark coloured, horny and brittle, though there was little change in dry weight.

SUMMARY

1. The aneurin content of the scutellum and embryo portion of the germ of various wheat types and of rye, barley, oats and maize has been determined.

2. Expressed as an average for all the wheats, the scutellum was 1·5% of the grain weight and contained 59% of the total aneurin in the grain, whilst the embryo was 1·2% of the grain weight and contained 3% of the total vitamin. Corresponding figures for the other cereals were of the same order.

3. No loss of aneurin from the scutellum could be detected in wheat which had been soaked in water for up to 12 hr.

4. Analytical figures relating to total N, total P, phytate P, phosphatase and petrol ether extract for scutellum and embryo of wheat are given. The scutellum contained 1·3% phytate P, which was more than three times that of the embryo, and 30% lipoid material, which was double that of the embryo.

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The Metabolism of Azo Compounds

1. AZOBENZENE

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B. Fischer (1906) described epithelial proliferation following the injection of the azo dyestuff scarlet red. Although the growths were non-malignant he rightly claimed his discovery as the first case of the induction of tumour-like proliferation by a chemical compound. Hayward (1909) found that the active part of the scarlet red molecule was represented by 4′-amino-2:3′-azotoluene, and Yoshida (1934) produced proliferation of liver tissue in mice and liver tumours in rats with this substance. Kinosita (1937) later examined other derivatives of azobenzene and found *p*-dimethylamino-azobenzene to be highly active in producing liver tumours; and bladder tumours were produced with 2:3′-azotoluene.

In other attempts to produce bladder cancer, Cook, Hewett, Kennaway & Kennaway (1940) selected the three azonaphthalenes, on the supposition that these might arise by oxidation of naphthylamines and so be a factor in the occupational incidence of the disease in chemical workers. No bladder tumours were obtained, but liver changes in mice, similar to those induced by the azobenzene derivatives, resulted from treatment with 2:2′-azonaphthalene. To explain the carcinogenic action of this compound they suggested that it might be reduced in the body to 2:2′-hydrazonaphthalene, which, by a 'benzidine' type of rearrangement is converted to 2:2′-diamino-1:1′-dinaphthyl, and subsequently to 3:4:5:6-dibenzcarbazole, which may be the real carcinogenic agent. This suggestion is supported by the fact that 2:2′-diamino-1:1′-dinaphthyl was itself found to be capable of inducing liver tumours in mice and 3:4:5:6-dibenzcarbazole had been shown to produce skin cancer and malignant connective tissue tumours as well as liver tumours (Boyland & Brues, 1937; Andervont & Edwards, 1941). It thus provides a remarkable linkage between carcinogenic substances of the azo class, and the polycyclic compounds.

No direct evidence that such metabolic changes can take place in the body has, however, hitherto been produced, and we therefore began a systematic study of the metabolism of azo compounds, starting with azobenzene, the simplest member of the group.

METHODS

Administration to rats. Twenty adult male rats were given 50 mg. azobenzene, dissolved in 0·5 ml. arachis oil, by intraperitoneal injection. As this appeared to be well tolerated the dose was increased to 75 mg. for subsequent injections (100 mg. azobenzene had been found previously to be toxic, causing death of a number of the animals). The injections were repeated every 3 or 4 days, the animals being kept in metabolism cages and the urine collected.

Colorimetric estimation of amines. Amino metabolites were estimated in the urine by a colorimetric method based on the colours obtained by coupling the diazotized amines with *N*-sulphatoethyl-*m*-toluidine, a reagent which has been found by Rose & Bevan (1944) to have advantages over the usual coupling components in sulphanilamide estimations. The colours obtained were compared by means of a Hilger 'Spekker' Photoelectric Absorptiometer, and by the use of Ilford 'Spectrum' filters rough absorption curves were obtained.

Reagents. (1) $NaNO_2$, 0·1%. (2) Sodium acetate, $C_2H_3O_2Na.3H_2O$, 20%. (3) *N*-sulphatoethyl-*m*-toluidine, 1%. All as aqueous solutions.

Method of estimation. 1 ml. of the urine is treated with 0·1 ml. of conc. HCl followed by 1 ml. of 0·1% $NaNO_2$. After 2 min., 2 ml. of 20% sodium acetate are added, 1 ml. of the *N*-sulphatoethyl-*m*-toluidine reagent, and 5 ml. of ethanol. After being mixed and allowed to stand for 10 min. for development of the colour (usually yellow at this stage), the solution is acidified by addition of 0·5 ml. conc. HCl, giving the red colour which is estimated on the colorimeter.

For determination of colour in the urine after hydrolysis, 6 ml. of the urine were treated with 1 ml. conc. HCl and hydrolyzed by gentle boiling for 5 min. The precipitate which formed was removed by centrifuging and the colour estimation carried out as described on 1 ml. of the clear supernatant liquid.

RESULTS

The urine from the azobenzene-treated rats, when diazotized and coupled with *N*-sulphatoethyl-*m*-toluidine, gave an intense red purple colour from which a purple precipitate soon separated out. Neither aniline nor *p*-aminophenol gave this purple precipitate, and moreover the purple colour was found to dye cotton, for which aniline type azo dyes have no affinity. Benzidine was found to give a coloured substance with the coupling reagent, which was identical in properties with that obtained from the urine.

Extraction of the urine. The urine (750 ml.), which was alkaline to litmus, was extracted with ether in a continuous extractor for 40 hr. The ether extract, after evaporation of the ether, was treated with HCl (1 ml. of conc. HCl diluted with 5 ml. water). Some oil remained undissolved and this was separated by filtration, with the aid of a little charcoal. After washing with water the volume of the filtrate was 20 ml. 0·1 ml. of this solution subjected to the colour test gave a red colour for which a colorimeter reading E_{green} 1·5 was obtained. On standing, a small amount of purple precipitate separated out. The remainder of the solution was made alkaline

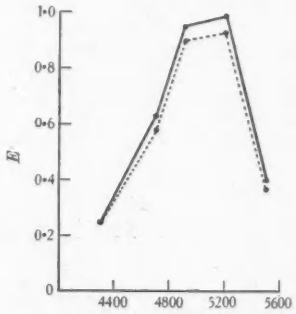


Fig. 1. Absorption curves obtained by the use of the 'Spekker' photoelectric absorptiometer on the colours obtained from the steam distillate of the ether extract of the urine of rats treated with azobenzene, and from aniline. —— Steam distillate from ether extract of azobenzene urine. ----- Aniline (0·025 mg.)

with NaOH and steam distilled. The distillate gave a bright red colour reaction, 0·15 ml. of the first runnings giving the colorimeter readings shown in Fig. 1, which includes for comparison the colour

readings obtained with aniline, when diazotized and coupled with *N*-sulphatoethyl-*m*-toluidine under identical conditions. There is very close agreement, the curves for aniline and the metabolic product being practically identical.

Steam distillation was continued until the distillate gave only a faint colour reaction. 0·25 ml. of the total distillate (330 ml.) gave a colorimeter reading E_{green} 0·85, from which it was estimated that the total amount of aniline present was about 33 mg.

The residual alkaline urine after this first ether extraction was acidified with conc. HCl (125 ml.) and heated on the steam bath for 1 hr. After removal by centrifuging of the precipitate which formed, the clear liquid was made alkaline with NaOH (50 g. dissolved in a little water) and again extracted with ether in the continuous extractor for 40 hr. The ether extract, after removal of the ether, was boiled with a little water with addition of a small amount of charcoal and filtered. On cooling, crystals separated from the filtrate. These were filtered off and recrystallized from water. After drying at 110° the m.p. was 123–125°; mixed m.p. with benzidine (m.p. 127°) gave m.p. 124–126°.

Sulphur and glucuronic acid estimations

Quantitative estimations of the daily inorganic sulphate, ethereal sulphate, neutral sulphur and of the glucuronic acid excretion were carried out. For the glucuronic acid estimations the method of Maughan, Evelyn & Browne (1938) was used and the sulphur estimations were carried out by a modification of the usual benzidine method, details of which will be published later.

Ten young male rats were kept on a standard diet for a week before treatment with azobenzene, urine being collected daily and S and glucuronic acid estimations being carried out. The animals then each received 50 mg. azobenzene by intraperitoneal injection and the daily estimations of S and glucuronic acid were continued. The results are given in Table 1.

Table 1. *Excretion of* S *and of glucuronic acid by rats before and after administration of azobenzene*

Results are expressed as mg. of S or of glucuronic acid/100 g. rat/day.

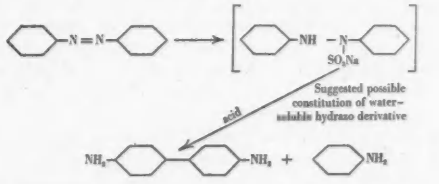
Days after injection	Inorganic S	Ethereal S	Neutral S	Glucuronic acid
Control period. Ten male rats each given intraperitoneally 1 ml. arachis oil				
1	1·4	0·18	0·48	7·5
2	2·9	0·28	0·78	9·4
3	3·0	0·34	0·64	13·0
Experimental period. The above ten rats each given intraperitoneally 50 mg. azobenzene in 1 ml. arachis oil				
1	1·3	0·32	1·7	14·5
2	0·1	1·40	1·8	31·4
3	3·8	0·95	4·3	35·4
4	6·6	0·27	2·5	25·0

The injection of azobenzene results in a marked rise in the ethereal sulphate and in the glucuronic acid excreted, maximum values being obtained on the second and third days after injection. The amount of amine excreted as measured by the colour reaction also attains a maximum during this period. A considerable increase in the excretion of neutral sulphur occurs, reaching a maximum on the third day. A similar increase in neutral sulphur excretion has been observed in rats treated with the carcinogenic *p*-dimethylamino-azobenzene and this is being further investigated.

DISCUSSION

From the urine of rats injected with azobenzene the products which have actually been isolated are aniline and benzidine. By ether extraction of the unacidified urine (which was slightly alkaline) only aniline was identified in the extract, the colour reaction indicating the presence of only a minute trace of benzidine. The benzidine which was subsequently isolated must therefore be derived from a water-soluble product, present in the original urine, but not extractable with ether. On acidification with dilute acid, benzidine is formed from this water-soluble product and, after the solution has been made alkaline, can readily be extracted with ether.

To obtain benzidine from azobenzene, reduction to hydrazobenzene must first take place, and to explain the above facts we suggest that this reduction takes place in the body, the hydrazobenzene being then rapidly excreted as a water-soluble derivative, formed possibly by conjugation with sulphuric acid as a *N*-SO_3Na derivative, or with glucuronic acid in a similar manner.



This derivative on treatment under fairly mild acid conditions is assumed to undergo the benzidine rearrangement. It is possible that this rearrangement can take place to some extent *in vivo*, particularly in certain organs such as the liver. In fact the liver of azobenzene-treated rats, after being heated with alkali and extracted with ether, yielded an extract which gave a faint colour reaction for

benzidine. The amount present in the liver must be very small, however, and it is evident that the main tendency is for azobenzene to be rapidly eliminated as the water-soluble hydrazo derivative.

Azobenzene is not carcinogenic, but in work which is being carried out with the carcinogenic *p*-dimethylamino-azobenzene evidence has been obtained that similar metabolic changes take place. In this case, however, the hydrazo derivative does not appear to be so stable and undergoes the benzidine rearrangement in the body more readily, since a substance giving the colour reaction of one of the 'benzidine change' products of *p*-dimethylamino-azobenzene can be extracted directly from the neutral or alkaline urine.

SUMMARY

The urine of rats which have received azobenzene has been found to contain aniline, and a water-soluble derivative which, after acidification with dilute acid followed by addition of sodium hydroxide and subsequent extraction with ether, yields benzidine.

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Changes in the Skeleton during Gestation and Lactation in the Rat

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Many observations show that during pregnancy mammals store Ca and P in the skeleton and that these substances are released during the ensuing lactation (Forbes, Black, Braman, Frear, Kahlenberg, McClure, Swift & Voris, 1935; Coons, Schiefelbusch, Marshall & Coons, 1935; Duckworth & Warnock, 1942–3; Goss & Schmidt, 1930). The extensive growth of cancellous bone which takes place during the pre-ovulatory stage with subsequent resorption about the time of ovulation in the pigeon suggests that a similar process occurs also in birds (Kyes & Potter, 1934; Bloom, Bloom & McLean, 1941). The classical studies of Bauer, Aub & Albright (1929) showed that cancellous bone served as a store of Ca and P and that storage and depletion were governed by the level of Ca intake. Using alizarin red as an intravital stain, they also demonstrated that mobilization of the trabeculae was caused by parathyroid hormone.

Apparently, no investigation has been specifically designed to determine if the trabeculae are the sole physiological store of Ca and P in the adequately nourished animal, or whether, in lactation, the shaft is also drawn upon by direct resorption, or by partial demineralization without resorption, or by resorption with concomitant partial demineralization of the unresorbed portion. The importance of this fundamental question is underlined by the description given by Theiler & Green (1931–2) of the modern, high milk-producing, dairy cow: 'such an animal is pathological, oscillating between a compact bone tissue and a mildly osteoporotic framework in each reproductive cycle.' In addition no comparison seems to have been made of the behaviour of the skeleton of animals lactating normally with that of animals which, at the conclusion of gestation, are not allowed to suckle their young. Neither have comparative studies been made of the rates of replenishment of skeletal stores after lactation in pregnant and non-pregnant animals.

The present investigation was designed to examine these points. The shafts of certain long bones were separated from the ends and the two portions studied. Although this mode of approach has obvious limitations, it permitted some clarification of the problem.

EXPERIMENTAL

(*a*) *Groups of animals*

One hundred and fifty female hooded Lister rats were divided into twenty-five subgroups of six rats each and these subgroups were distributed among the three groups described below. All rats were transferred to the experiment on reaching 100 days of age. Litter mates were distributed evenly among the three groups, being placed in appropriate corresponding subgroups. This plan was not strictly followed, for some rats failed to conform to the standard of performance given below; they were discarded and replaced by others.

The three groups into which the animals were divided were treated as follows:

Mated-rat group. These rats were introduced to the male at 100 days of age and the occurrence of coitus was detected by vaginal smear. Of the nine subgroups three were killed during the first gestation, one at the seventh day (reckoned from the date at which either a vaginal plug was seen or remains of spermatozoa were found in the smear), one at the fourteenth day and one at parturition. Six subgroups began lactation, one being killed on the seventh day, one on the fourteenth day and one on the twenty-third and final day of lactation. The remaining three subgroups were reintroduced to the male at the end of lactation, tested for coitus and killed at the same intervals as in the first gestation.

Control Group A. Of the six subgroups in this group three served as non-lactating controls to those lactating in the mated-rat groups. The rats of these subgroups were mated as in the above group but their litters were killed at birth. One subgroup was killed on the seventh day after parturition, one on the fourteenth day and one on the twenty-third day. The rats of the remaining three subgroups suckled their young but were not re-mated; they acted as controls for the animals in the mated-rat groups during the second gestation. They were killed at times corresponding to those in the second gestation of the mated-rat group.

Control Group B. The rats in this group served as non-pregnant and non-lactating controls of the mated-rat group. They were sacrificed at the same time as their corresponding litter-mates in that group. This group also included a subgroup, the rats of which were killed at 100 days of age.

The normal stock diet of the colony was used and consisted of feeding cubes (Thomson, 1936), greenstuff (cabbage, kale or clover), 5 ml. fresh milk daily and tap water. The percentage composition of the feeding cubes was: wheat offal (fine bran), 17·76; ground wheat, 17·76; Sussex ground oats, 17·76; ground barley, 8·80; ground maize, 8·80; meat and bone meal (45% protein), 8·80; dried skimmed milk, 14·00; white fish meal (60% protein), 4·30; dried yeast (40% protein), 1·10; sodium chloride, 0·46; cod-liver oil, 0·46. With the start of the experiment the milk allowance of all rats was increased to 10 ml. daily, with a further increase to 25 ml. during lactation. The animals were housed in screen-bottomed, galvanized iron cages with wood wool as bedding. They were weighed every third day and killed at the appropriate time with coal gas. Litters were reduced to eight in number on the day following parturition and were weaned at 23 days of age.

Rats were discarded and replaced by others if: (1) their clinical examination revealed any abnormality such as respiratory disorders, (2) when mated they failed to become pregnant within two oestrus cycles, (3) their litters contained less than eight living young, (4) of the eight young ones left with the mother any died during lactation.

(*b*) *Methods*

After dissection from the body the hind legs were immersed in boiling water for 3 min. to facilitate cleaning. The cleaned bones were then repeatedly extracted with boiling ethanol to remove fat.

Both tibiae and femora were used for analyses. One-quarter of the length of each femur was measured from each end and these portions sawn off with a jeweller's file. The loss in bone dust due to sawing is small and causes no serious errors in analysis. The tibia was divided so that one-quarter of its length, measured from the proximal end, and one-tenth of its length, measured from the distal end, could be sawn off. The shafts were opened and the cancellous bone and marrow within the cavity was added to the eight ends from the tibiae and femora in order that, as far as possible, the shafts should include only compact bone and that cancellous bone should be included in the composites subsequently referred to as 'ends'. The remainder of the bones, subsequently called 'shafts', thus represented half the length of the femora and thirteen-twentieths of the length of the tibiae.

The ends and shafts were dried in an oven at 99° to constant weight. The ashing was carried out in an electric furnace at dull red heat until weight was constant.

RESULTS

The curves in Figs. 1–3 were obtained by joining together the average values of the body weight and bones observed at death in the subgroups. In discussion they will be referred to as growth curves. It should be noted that of the six subgroups of control A rats, three served as controls during the lactation period and three as controls during the second gestation and therefore the points on the curve at the twenty-third day of lactation and the seventh day of the second gestation could not be joined.

Fig. 1 shows the degree of variability in the average body weights of the different subgroups. Comparison of groups and subgroups should be made with these variations in mind, particularly in the case of minor differences. Certain points, outside the scope of the present study and technique, are investigated more satisfactorily by the metabolic balance method.

In Fig. 2 the weights of the dry, fat-free ends and shafts of the bones, and of whole bones are recorded. The values for whole bone were obtained by combining the corresponding values for ends and shafts. During the first gestation period the weights of whole bones of the mated rats were slightly greater than the weights of those of control B rats; the rates of increase in bone-weight were similar. With the onset of lactation there was a sharp reduction in the weight of whole bones of the mated-rat groups which continued in an almost linear manner throughout lactation. In the second gestation there was a

sharp increase, again almost linear, in the bone weights of the mated-rat groups. Throughout the study the bone weights of the control B rats increased in a somewhat irregular manner owing to

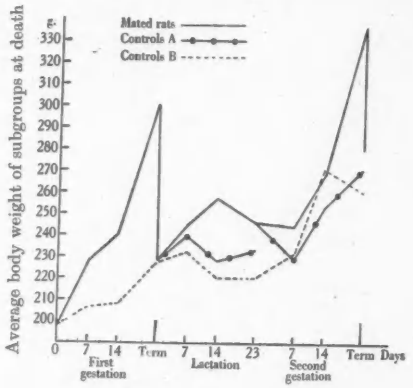

Fig. 1. Average body weight of each subgroup at the time of killing, showing variation in body weight within groups. —— Mated-rat group. —●— Control group A. ---- Control group B, non-pregnant, non-lactating rats.

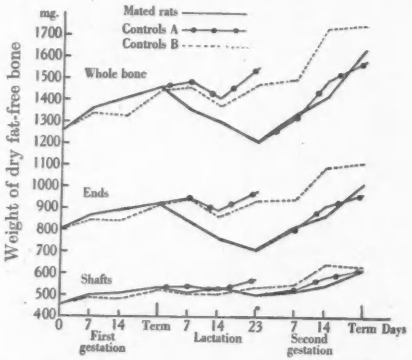

Fig. 2. Average dry fat-free weight, in mg., of the shafts, ends and whole bone (shaft and ends) of each subgroup at different stages of gestation and lactation. The femora and tibiae were used. —— Mated-rat group. —●— Control group A. ---- Control group B, non-pregnant, non-lactating rats.

differences in body weight between subgroups, as is shown in Fig. 1. It is, of course, obvious that no comparison on a body weight basis can be made of the bone data of pregnant or lactating rats with non-pregnant or non-lactating rats. The bones of the control A rats, whose litters had been killed at birth, behaved in a manner similar to those of control B rats during the interval corresponding to lactation in the mated-rat group. The greater weights of control A rats as compared with control B rats during this period may be due to the previous pregnancy. After lactation the control A rats showed a rate of bone growth which was almost identical with that of the mated-rat groups undergoing the second gestation. The total weight of dry fat-free bone was greater in the mated-rat group at the end of the second gestation than at the end of the first gestation. From the end of the first gestation the whole bones of the control B group were consistently heavier than those of the mated-rat groups.

The changes observed in whole bone weight during lactation and the second gestation reflected the changes which occurred in the bone ends. As measured by weight, shaft growth continued during the pregnancy of the mated rats, and may have undergone a change during lactation. Compared with the B control rats, the mated rats showed during their second gestation a decrease in bone weight. This decrease, also seen in the non-pregnant A rats, may have resulted from a slackening of bone growth during lactation. There is, of course, no evidence of a resorption of shaft in any way comparable with that occurring at the bone ends.

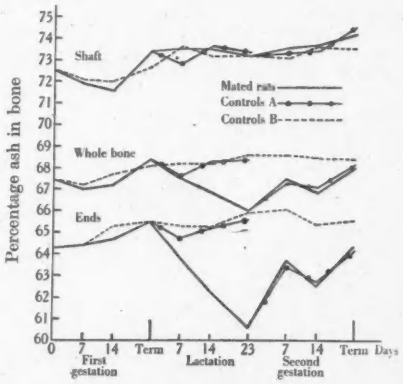

Fig. 3. Percentage ash in dry fat-free shafts, ends and whole bones (ends and shafts) of each subgroup at different stages of gestation and lactation. The femora and tibiae were used. ——Mated-rat group. —●— Control group A. ---- Control group B, non-pregnant, non-lactating rats.

The values for the weight of ash in whole bones, ends and shafts followed paths almost identical with those of dry fat-free bones. The changes in ash content of the whole bones and ends were, however, exaggerations of those found for dry fat-free bone, with the result that the percentage ash values did not remain constant, as is shown in Fig. 3.

The percentages of ash in the shafts of the rats of all groups were not significantly different, and were remarkably constant after an initial reduction followed by an increase. In the whole bone of the mated-rat group a reduction in the percentage of ash during lactation resulted from changes in the percentage of ash in the bone ends. During the interval corresponding to lactation in the mated-rat group the percentages of ash in the whole bones and ends of the control A rats followed the path of the values for control B rats. The reduction observed at the seventh day in the non-lactating control rats is difficult to explain, but since it occurred equally in ends and shafts it may have arisen from causes not connected with the experiment.

The changes in the percentage of ash in the ends of the bones from the seventh to fourteenth day of the second gestation were unexpected. They occurred in both the non-pregnant rats of control A group and in the pregnant rats of the mated-rat group. The percentage of ash in the bones of both groups followed almost identical paths. The final value for percentage ash in the ends did not again reach the high level at the termination of the first gestation although the total amount of ash in the ends was greater. The percentage ash in the whole bones, however, came close to the term value of the first gestation, because of increases in the percentage of ash in the shaft during the second gestation.

DISCUSSION

The present technique, although suitable for the purpose of this study, is not refined sufficiently for the detection of slight differences. The amount of minerals deposited in the skeleton during pregnancy in preparation for the forthcoming lactation is small in relation to the weight of the skeleton and even to the weight of long bones, in which the principal storage occurs. Although the present study indicated storage during gestation, too much reliance should not be placed in the technique used as a means of investigating such problems. It must be noted that at 100 days the skeleton of the female rat has not completed its own growth and that the observations might have been different if very young or mature breeding females had been used. However, the data do warrant the conclusion that mineral deposition during pregnancy in the rat proceeds at a rate which is at least normal and that this continued growth is not restricted to the spongiosa, but extends to the shaft as well.

The sharp reduction in the weight of the whole bone, with an even greater reduction in the total quantity of ash, that begins with and continues throughout lactation was restricted to the bone ends. There is, perhaps, some indication that shaft growth is suspended during lactation, but there was no evidence of resorption or demineralization without resorption in any way comparable with the changes in bone ends.

The total evidence in this study indicates that two processes operate in the bone during lactation: resorption and demineralization of the unresorbed residue. Resorption requires no further discussion beyond calling attention to the presence of considerable quantities of cortex in the samples referred to as ends. If the corticalis in the epiphyseal and metaphyseal regions has the same composition as that in the diaphyseal zone, the changes in the spongiosa must have been correspondingly greater.

In studies of the bones of pigeons during the laying period and under the influence of toxic doses of parathyroid hormone, Bloom & Bloom (1940), Bloom *et al.* (1941) and McLean & Bloom (1940, 1941), found that bone resorption occurred and that the organic matrix and bone salt disappeared simultaneously without preliminary demineralization or the formation of osteoid borders. If histological staining techniques can detect partial demineralization processes physiologically distinct from resorption, the reductions observed in the percentages of ash during lactation could only have arisen from an accumulation of non-matrix organic matter within the bone cavity, which increases the volume as the spongiosa recedes.

On the other hand, the changes in the percentages of ash during the first and second week after the end of lactation could be explained easily if partial demineralization in addition to resorption had occurred during lactation. While the number of rats in each group is comparatively small, it should be noted that after lactation almost identical trends were found in both pregnant and non-pregnant rats. Thus, if, after lactation, minerals were deposited in the partially demineralized bone as well as being utilized for new bone growth, the rate of mineral deposition relative to the organic matter deposited would be greater in the earlier stages.

The behaviour of the bones of the non-lactating rats whose litters had been killed at birth indicates that the changes occurring in the lactating rats were a consequence of lactation and not of pregnancy or parturition. No endocrine mechanism intervenes to cause liberation of the mineral stores of pregnancy if they are not required subsequent to parturition.

The almost identical rates of replenishment of skeletal reserves in the pregnant and non-pregnant rats, during the period when the rats of the mated group were undergoing their second gestation, indicates that, within the limits of the present method, pregnancy did not exert a marked influence on storage. Since the pregnant rats were depositing minerals in the foetuses at this time, there was, of course, a greater total storage.

It is clear that the bone shafts of the lactating rat with a history of good nutrition, as in this study,

are not used as a source of minerals to sustain the mammary supply during lactation. In other words, an osteoporotic skeleton is not an obligatory consequence of lactation in this species at least. Considerable difficulties exist in the determination of these requirements by the metabolic balance technique, since this method does not indicate when mobilization of minerals from the spongiosa is accompanied by mobilization from the shaft. Further, the balance over a whole lactation is often small in relation to the total quantities of materials analyzed and may be close to the analytical errors of the methods when the diet given is rich in minerals. The present observations may find some application in the determination of certain mineral requirements of lactation. Because the losses of Ca and P are unavoidable, involving the gradual liberation of reserves laid down at an earlier period, it has been difficult to estimate the requirements for lactation on the basis of the negative values obtained by the balance technique. Since the present study shows that, in the rat, it is possible to restrict the withdrawal of bone salt to the spongiosa, leaving the shaft unaffected, it seems reasonable to suggest that lactation requirements could be defined as those intakes which, for a given output of minerals in milk, will restrict the withdrawals to the spongiosa. Thus, using a similar technique, the problem of the mineral requirements for lactation in domestic animals might become more amenable to experimental attack. This preservation of the diaphyses may be an unattainable ideal in the high-producing dairy cow, but in sheep and swine it may be possible as in the rat. Also since Theiler (1934) pointed out that, in cattle, P deficiency had a more marked effect on the skeleton than Ca deficiency, the method might be more suitable for the determination of P than Ca requirement in the cow. While no evidence has yet been produced to show that a mild degree of osteoporosis is seriously detrimental to the individual, its avoidance would preserve the value of the skeleton from a structural standpoint.

SUMMARY

1. A technique for studying changes in the ends and shafts of the femur and tibia of the rat is described and used to follow the effect of gestation and lactation upon bone.

2. The percentage ash content and weight of dry, fat-free bones of well-fed rats were determined. During lactation and pregnancy the changes which occurred were confined to the bone ends.

3. The resorption of the spongiosa which occurred in lactating rats was not observed in non-lactating animals. The rate of replenishment of the depleted spongiosa at the end of lactation was the same, irrespective of whether the animals were then pregnant or non-pregnant.

4. The shafts of the bones of rats were little affected during lactation. The use of this finding in the determination of the Ca and P requirements of domestic animals is discussed.

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Qualitative Analysis of Proteins: a Partition Chromatographic Method Using Paper

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Gordon, Martin & Synge (1943*b*) attempted to separate amino-acids on a silica gel partition chromatogram, but found it impracticable owing to adsorption by the silica of various amino-acids. They obtained, however, good separations by using cellulose in the form of strips of filter paper. Following further work along these lines, the present paper describes a qualitative micro-analytical tech-

nique for proteins. Using only 200 μg. of wool, it is possible by this method to demonstrate the presence of all the amino-acids which have been shown to be there by other methods.

The method is rather similar to the 'capillary analysis' method of Schönbein and Goppelsroeder (reviewed by Rheinboldt, 1925) except that the separation depends on the differences in partition coefficient between the mobile phase and water-saturated cellulose, instead of differences in adsorption by the cellulose. That adsorption of the amino-acids by the cellulose plays no significant part is seen from Table 1, where the partition coefficient calculated from the rates of movement of the bands are compared with those found directly by England & Cohn (1935). Too much stress should not be laid upon the agreement of these figures, which are based upon an assumed water content of the saturated cellulose and the assumption that the ratio of the weight of *n*-butanol to paper is constant in all parts of the strip. This assumption does not hold accurately. Nevertheless, the conclusion seems justified that the cellulose is playing the role of an inert support.

The most satisfactory solvents are those which are partially miscible with water. Within a homologous series of solvents the corresponding rates of movement of the amino-acids change in the same direction as the water solubility of the solvent: solvents completely miscible with water can be employed provided that the water content is not too high. In this case, presumably, the cellulose, by a 'salting out' effect, allows the system to function as a partition chromatogram. However, the amino-acid bands obtained are much broader than is the case with immiscible solvents. This is no doubt due to a variation in the composition of the phases caused by the presence of the amino-acids. This effect has been noticed in the *n*-butanol-water system by England & Cohn (1935) and is the limiting factor in the amount of amino-acid that can be employed in a given chromatogram. It is reasonable to suppose that the phases will be more easily disturbed by employing a miscible rather than an immiscible solvent. The main effect of temperature on the rates of movement of amino-acids is also explicable in terms of the change in composition of the phases. Thus, in the phenol-water system, increase of temperature increases the miscibility and the rate of movement of the bands. In the collidine-water system the reverse is true. Further, the greater the difference between the working temperature and the critical solution temperature, the less sensitive will the rates be to change of temperature. However, though the absolute partition coefficients may be greatly changed, the ratios of the partition coefficients of the respective acids are almost unaltered.

There is an obvious advantage in working with unsubstituted amino-acids in that any substituent group, even though small, renders the physical properties of the derivatives more similar and hence increases the difficulties of separation. Martin & Synge (1941) and Gordon, Martin & Synge (1943*a*) failed to separate the slower moving acids when acetylated, whereas all the acids are separable by the present technique. Moreover, selective losses associated with acetylation and extraction are obviated.

A considerable number of solvents has been tried. The relative positions of the amino-acids in the developed chromatogram depend upon the solvent used. Hence by development first in one direction with one solvent followed by development in a direction at right angles with another solvent, amino-acids (e.g. a drop of protein hydrolysate) placed near the corner of a sheet of paper become distributed in a pattern across the sheet to give a two-dimensional chromatogram characteristic of the pair of solvents used. Advantage is taken of the colour reaction with ninhydrin (see Copley, 1941, for bibliography) to reveal the positions of the amino-acids.

Relation of partition coefficient to rate of movement of bands

The relation of the partition coefficient to the rate of movement of the band may be calculated by the method of Martin & Synge (1941).

A = cross-sectional area of paper + water + solvent,

A_L = cross-sectional area of solvent phase,

A_S = cross-sectional area of water phase,

α = partition coefficient

$$= \frac{\text{concentration in water phase}}{\text{concentration in solvent phase}},$$

$$R = \frac{A}{A_L + \alpha A_S}.$$

However, R is not conveniently measurable in paper chromatograms, so a new symbol, R_F is introduced.*

$$R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}} = \frac{RA_L}{A} = \frac{A_L}{A_L + \alpha A_S}$$

or
$$\alpha = \frac{A_L}{R_F A_S} - \frac{A_L}{A_S} = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right).$$

A_L/A_S is equal to the ratio of the volumes of solvent and water phase in the chromatogram.

* The symbol R_F is equivalent to the symbol R used by LeRosen (1942) who apparently received the paper of Martin & Synge (1941) too late to avoid using the symbol R in a different sense.

Table 1. *Partition coefficients calculated from R_F values*

No. of run	1	2	3	4	Direct measurements England & Cohn (1935)
% water in paper	28·7	18·0	22·6	17·7	
A_L/A_S	3·25	4·56	3·70	2·93	
Partition coefficients:					
Glycine	70·4	70·4	70·4	70·4	70·4
Alanine	35·9	39·9	43·7	36·6	42·3
Valine	12·2	14·1	14·8	12·5	13·8
*Nor*valine	8·7	10·8	10·5	9·2	9·5
Leucine	4·5	5·4	5·6	6·0	5·5
*Nor*leucine	3·5	4·2	4·4	4·6	3·2

Assuming a given water content of the paper, A_L/A_S may be deduced from the ratio of weight of dry paper to that of the developed chromatogram.

The water content of the paper is apt to vary from experiment to experiment and is difficult to measure in the presence of *n*-butanol or other solvent. Table 1 shows the partition coefficient calculated from the R_F and A_L/A_S values for four separate runs under slightly different conditions. The water content has been so chosen that the partition coefficient for glycine is equal to that given by England & Cohn (1935). The last column gives the direct measurements of England & Cohn.

The water content of saturated cellulose, according to the *International Critical Tables*, is 22 % on a dry-weight basis.

EXPERIMENTAL

The essentials of the apparatus consist of a filter-paper strip, the upper end of which is immersed in a trough containing the water-saturated solvent. The strip hangs in an airtight chamber in which is maintained an atmosphere saturated with water and solvent. The trough is provided with a bar over which the paper passes to prevent capillary siphoning between the outside of the trough and the paper. The apparatus is shown in Figs. 1–4.

Chamber. For one-dimensional experiments, stoneware drain-pipes (6 or 9 in. in diameter and 2 ft. and 2 ft. 6 in. long respectively) have been found convenient. The pipe stands in a close-fitting tray hammered from a sheet of lead. The top of the pipe is ground flat and covered by a sheet of glass (Fig. 3). The glaze on these pipes is imperfect and a pipe should be reserved for each solvent and atmosphere. For two-dimensional experiments, the chamber consists of a glass-sided lead box, 30 × 30 × 5 in., made airtight with a well-fitting lead lid. A removable lead tray rests on the floor of the box. A completely air-tight tinplate box, using the tray as a water seal, was used when an atmosphere of coal gas was required.

Troughs. The troughs used in the one-dimensional experiments are constructed from ⅜ in. bore glass tubing, opened along its length by grinding (Figs. 1, 2). For two-dimensional work, the larger troughs required would be too fragile if of the same pattern. Therefore an iron frame supports the trough (of ½ in. bore thick-walled tubing) and carries strips of sheet glass which replace the bars. The cross-section is shown in Fig. 4.

Paper. Whatman no. 1 filter paper has been used almost exclusively. Whatman no. 42, while giving satisfactory separations of amino-acids, was too dense to permit a convenient rate of flow of solvent. In an attempt to separate larger quantities of amino-acids, thicker papers have been tried. Of these, Whatman 'Accelerator' paper (1/75 in. thick) gave satisfactory separations but was too fragile to withstand normal handling. Whatman 'Seed Testing' paper (1/75 in. thick) was unsatisfactory as uniform flow of solvent could not be obtained. With most solvents the advancing front of liquid is coloured yellowish brown by material extracted from the paper. This contaminant can be partially removed by Soxhlet extraction with ethanol. Chromatographic washing is more effective. However, as the contaminant is so fast-running, relative to the amino-acids in most solvents (except phenol), it therefore does not interfere, and unextracted paper has usually been used.

Procedure

To run a one-dimensional chromatogram a strip of paper, 1·5 cm. or more in width and 20–56 cm. in length, is used. A pencil line is drawn across the strip about 5 cm. from one end. The solution, 2–4 μl. containing 5–15 μg. of each amino-acid to be analyzed, is applied along the centre portion of this line from the tip of a capillary tube. The end of the paper is fixed in the trough with a microscope slide. The trough and paper are now transferred to the chamber, which has been previously prepared by covering the bottom of the tray with a two-phase mixture of water and solvent to provide an atmosphere saturated with both components. The trough is filled with the water-saturated solvent and the lid put on the chamber. When the solvent has run a convenient distance (15–25 cm. in 6 hr.; 30–50 cm. in 24 hr., depending on solvent and temperature), the paper is removed and the position of the solvent front is marked. The strip is dried, either in an oven at 110° or by hanging in a drying cupboard through which hot air is sucked by a fan exhausting to the outside. After drying, the paper is sprayed with a solution of ninhydrin (0·1 % in *n*-butanol) and again dried. Finally, the paper is heated at 80° for 5 min. The bands are outlined in pencil, as fading of the colour takes place after a few days. When it is

Two-dimensional chromatogram of a wool hydrolysate (180μg.) on Whatman no. 1 sheet. Hydrolysate applied at circle. Run with collidine for 3 days in direction *AB*, then in direction *AC* with phenol for 27 hr. in an atmosphere of coal gas and NH_3 (produced from a 0·3% NH_3 solution). The filter employed in photographing renders the yellow proline spot scarcely visible. (Photography by J. Manby, photographer to the University of Leeds.)

Two-dimensional chromatogram of a mixture of the hydrochlorides of 22 amino-acids (6–12μg. of each) on Whatman no. 1 sheet, carried out as described under Pl. 1. As before, the yellow proline and orange hydroxyproline spots are not visible in the photograph. (Photography by J. Manby, photographer to the University of Leeds.)

desired to run a number of chromatograms simultaneously, the individual solutions may conveniently be placed side by side on a wide strip. It is seldom necessary to leave more than an interval of 1 cm. between the spots, but it is undesirable for the amino-acids to be too near the edge of the paper, as irregularities of flow are usually more pronounced there.

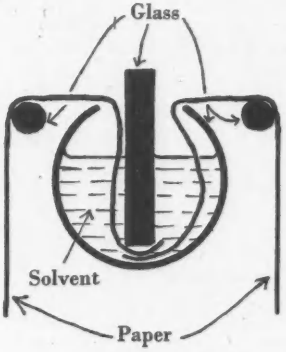

Fig. 1. Cross-section of small trough.

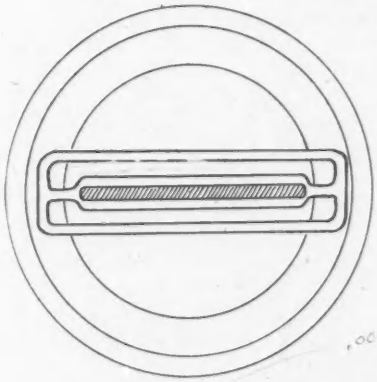

Fig. 2. Plan of small trough in pipe.

For two-dimensional analyses, a standard sheet $18 \times 22\frac{1}{2}$ in. is used (Pls. 1 and 2). The solution to be analyzed (6–12 μl., representing 200–400 μg. of protein) is placed near the corner, 6 cm. from either edge. The paper is held with one edge slightly overlapping the opening of the trough and pressed into it with a strip of sheet glass somewhat longer than the paper. After transfer to the chamber, prepared as above, the chromatogram is allowed to develop for 24–72 hr. The paper is then removed and dried in the drying cupboard, turned through a right angle, and returned to the trough. The next stage of development, again for 24–48 hr., now proceeds, the chamber, tray and trough having been prepared for the second solvent during the drying of the sheets. Subsequent treatment is the same as for the strips.

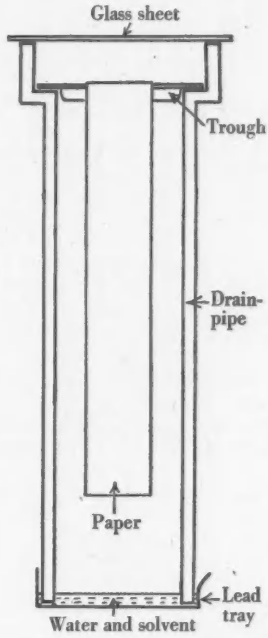

Fig. 3. Elevation of small trough in drain-pipe.

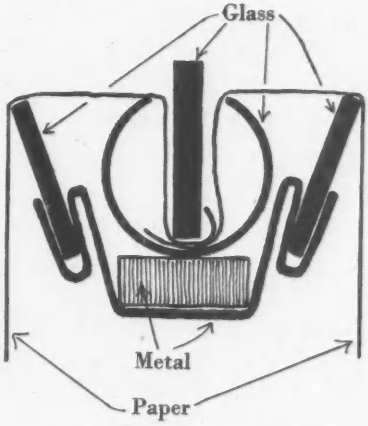

Fig. 4. Cross-section of large trough.

Table 2. *R_F values of amino-acid in various solvents on Whatman no. 1 paper, at room temperature*

Solvent ...	Phenol	Phenol	Phenol	Phenol	Phenol	Collidine	*n*-Butanol	*n*-Butanol	Tertiary amyl alcohol	Benzyl alcohol	1:1 *n*-Butanol benzyl alcohol	*o*-Cresol	*o*-Cresol	*m*-Cresol	*m*-Cresol	*p*-Cresol	*p*-Cresol	*Iso*-butyric acid
Addition ...	HCN	3% NH_3*	0·1% NH_3*	Coal gas	Cupron	—	Cupron	3% NH_3*	—	—	HCN	Cupron	Cupron + 0·1% NH_3*	Cupron	Cupron + 0·1% NH_3*	Cupron	Cupron + 0·1% NH_3*	—
Glycine	0·40 RP	0·40 RP	0·41	0·40	0·42	0·25 P	0·05	0·05	0·07 P	0·02	0·03 P	0·07	0·07	0·12 P	0·14 G	0·15 RP	0·19	0·36
Alanine	0·54 P	0·54 P	0·55	0·57	0·59	0·32 P	0·08	0·09	0·10 P	0·03	0·05 P	0·13	0·15	0·23 P	0·24 B	0·28	0·32	0·44
*Nor*valine	0·81 P	0·79 P	0·78	0·78	0·80	0·48 P	0·26	0·31	0·23 P	0·12	0·19 P	0·45	0·45	0·60 B	0·62 B	0·66 BP	0·66	0·71
Valine	0·77 P	0·76 P	0·76	0·78	0·77	0·45 P	0·20	0·22	0·18 BP	0·11	0·15 P	0·41	0·38	0·52 P	0·54 P	0·59 BP	0·60	0·65
*Nor*leucine	0·88 P	0·85 P	0·87	0·85	0·89†	0·60 P	0·47	0·51	0·42 P	0·27	0·36 P	0·72	0·67	0·80 B	0·78 P	0·82 BP	0·80	0·79
*Iso*leucine	0·86 P	0·81 P	0·87	0·82	0·86†	0·54 P	0·37	0·40	0·31 P	0·18	0·27 P	0·58	0·55	0·70 B	0·70 B	0·73 P	0·74	0·76
Leucine	0·85 P	0·83 P	0·86	0·84	0·88	0·58 P	0·38	0·46	0·36 P	0·21	0·31 P	0·61	0·62	0·73 B	0·73 B	0·73 BP	0·77	0·78
Phenylalanine	0·89 BP	0·87 P	0·90	0·86	0·93†	0·59 G	0·43	0·46	0·36 G	0·36	0·38 BG	0·81	0·74	0·82 GB	0·82 P	0·82 P	0·83	0·80
Tyrosine‡	0·64 GP	0·63 B	0·66	0·59	0·62	0·64 G	0·28	0·14	0·24 G	0·14	0·19 G	0·24	0·23	0·35 P	0·35 P	0·39 B	0·38	0·58
Serine	0·36 P	0·33 P	0·35	0·33	0·36	0·28 G	0·05	0·05	0·08 P	0·01	0·02 P	0·05	0·05	0·08 BP	0·08 B	0·11 P	0·13	0·34
Threonine	0·50 P	0·41 P	0·47	0·50	0·50	0·32 P	0·07	0·08	0·09 P	0·02	0·04 P	0·11	0·08	0·14 B	0·14 P	0·19 P	0·21	0·43
Hydroxy-proline	0·67 O	0·50 O	0·66	0·66	0·67	0·34 OY	0·07	0·05	0·10 O	0·04	0·05 O	0·27	0·23	0·32 GY	0·32 Y	0·40 BG	0·42	0·42
Proline	0·91 Y	0·85 Y	0·89	0·87	0·86†	0·35 Y	0·12	0·14	0·11 Y	0·12	0·12 Y	0·69	0·66	0·73 Y	0·75 Y	0·76 Y	0·78	0·57
Tryptophan	0·83 B	—	—	0·76	0·86	0·62 P	0·35	—	—	—	0·30 B	0·65	0·58	0·76	0·69	0·70 B	0·68	—
Histidine‡	0·69 RP	0·68 B	0·70	0·72	0·69	0·28 G	0·06	0·09	0·07 G	0·02	0·03 RG	0·25	0·33	0·34 P	0·44 GP	0·36 B	0·52	0·45
Arginine‡	0·59 P	0·89 P	0·85	0·67	0·62	0·16 P	0·03	0·05	0·02 P	0·01	0·01 P	0·02	0·44	0·07 P	0·76 P	0·12 BP	0·81	0·40
Ornithine‡	0·33 P	0·73 P	0·61	0·40	0·37	0·13 BG	0·01	0·03	0·02 P	0·00	0·00 P	0·01	0·21	0·03 P	0·47 P	0·04 P	0·59	0·24
Lysine‡	0·46 P	0·82 P	0·73	0·50	0·48	0·14 BG	0·01	0·03	0·02 P	0·00	0·01 P	0·01	0·20	0·04 P	0·54 P	0·07 BP	0·66	0·27
Aspartic acid ‖	0·15 BP	0·12 B	0·12	0·14	0·17	0·22 B	0·01	0·01	0·02 BP	0·00	0·00 P	0·00	0·00	0·01 RP	0·01 B	0·02 BP	0·02	0·31
Glutamic acid	0·25 P	0·13 P	0·19	0·24	0·28	0·25 P	0·01	0·01	0·02 P	0·00	0·01 P	0·01	0·01	0·01 P	0·02 B	0·03 BP	0·03	0·38
Lanthionine‡	0·20 RP	0·19 G	0·21	0·18	0·20	0·12 G	0·01	0·00	0·03 RG	0·00	0·00 P	0·01	0·01	0·01 R	0·03 RP	0·03 RP	0·05	0·21
Cystine‡	0·30 RP	0·24 YG	0·13§	0·13§	0·29	0·14 G	0·01	0·01	0·02 RG	0·00	0·00 P	0·02	0·01	0·04 P	0·04	0·02 RP	0·02	0·25
Methionine	0·80 P	0·76 P	0·83	0·82	0·81†	0·57 GP	0·26	0·05	0·27 P	0·17	0·21 P	0·58	0·49	0·64 B	0·63 P	0·71 BP	0·68	0·69

Colour given by ninhydrin = B, blue; G, grey; O, orange; P, purple; R, red; Y, yellow.

* The % figure refers to the strength of NH_3 solution present in quantity in the tray.
† These acids were applied to the paper after the solvent had already run some distance.
‡ These acids were put on as the hydrochlorides and neutralized with NH_3 before development.
§ Cystine is decomposed by these additions. R_F value refers to the decomposition product.
‖ Colour initially green. Only final colour shown in table.

Throughout the manipulations, care must be taken not to touch the paper with the hand as finger marks will show after heating with ninhydrin. Strips are handled with forceps, and sheets with special wide clips. For long runs, particularly overnight, it is desirable to lag the chamber, otherwise differences in temperature will cause water to distil from the tray, which may waterlog the paper and cause irregularity of flow.

When phenol is used, whether as first or second solvent, the faster moving bands are liable to distortion by the contaminant from the paper already mentioned. This trouble can be avoided by evenly spraying the top 5 in. of the strip or sheet with phenol before the trough is filled. In this way the contaminant is kept well ahead of even the fastest running amino-acids.

RESULTS

Rates of amino-acids in various solvents. R_F values for the most useful solvents are tabulated in Table 2. The colour given with ninhydrin is indicated by letters.

A number of other solvents has been tried and abandoned. Of these, ethyl acetate, methylethyl ketone, aniline, cyclohexanol, cyclohexanone, quinoline and 'light aniline fraction b.p. 174–181°' were unsatisfactory as the amino-acids ran too slowly, whereas with methyl acetate, acetone, *sec.*-butanol, pyridine, the picolines and the lutidines, the bands were either too fast or unduly broadened.

or basic amino-acids would be more soluble in the water phase. In the alkaline pH range, the R_F values of aspartic and glutamic acids would thus decrease and those of arginine, lysine and histidine increase and vice versa in acid solutions (cf. Sharp, 1939). This is indeed found with NH_3 and HCl in phenol and in *n*-butanol, whereas dilute acid or base have very little influence on the monoamino-monocarboxylic acids. The effect of NH_3 in various solvents can be seen in Table 2.

Hydrolysates, or mixtures in which the ratio of soluble inorganic salts to amino-acid is high, give unsatisfactory chromatograms. The salt attracts water from the atmosphere and the solvent and causes local waterlogging of the paper. The amino-acids are not readily washed from these regions and the resulting bands are grossly distorted. This salt effect may be eliminated by impregnating the paper with salt and using the solvent and atmosphere equilibrated with saturated salt solution instead of with water. However, the colours given with ninhydrin are fainter and redder than those usually obtained and the rates are considerably slower.

In view of the alterations of rates with HCl, it is essential that the hydrolysate should be neutralized with NH_3 vapour after the hydrolysate has been applied to the paper, since some of the amino-acids are insoluble in neutral solutions.

To avoid the presence of excess salt, care must be taken to remove HCl from hydrolysates as far as possible, by repeated distillation *in vacuo*.

Table 3. *The effect of temperature on R_F values*

	Phenol						
						Phase composition % phenol in	
Temp.	Aspartic acid	Glutamic acid	Glycine	Lanthionine	Cystine	Phenol phase	Water phase
7– 8°	0·18	0·31	0·41	0·20	0·25	74·2	7·6
16–18°	0·22	0·34	0·44	0·17	0·28	72·7	8·1
20°	0·21	0·28	0·42	0·21	0·27	72·1	8·45
25°	0·19	0·33	0·45	0·23	0·28	71·05	8·75

Table 4. *The effect of temperature on R_F values*

	Collidine									
									Phase composition % collidine in	
Temp.	Aspartic acid	Glutamic acid	Glycine	Alanine	Valine	Leucine	Serine	Proline	Collidine phase	Water phase
10–15°	0·22	0·25	0·25	0·32	0·45	0·58	0·28	0·35	41·7 (10°) 49·0 (15°)	7·8 (10°) 5·0 (15°)
20°	0·04	0·04	0·06	0·10	0·27	0·37	0·10	0·12	54·9	3·4
25°	—	0·04	0·05	0·10	0·18	—	0·07	0·08	59·3	3·0

Effect of temperature. The variations of rates with temperature in phenol and collidine are shown in Tables 3 and 4. The phase compositions are also shown (data from *International Critical Tables*).

Additions of acids, bases and salts. It is to be expected that the ionized forms of the dicarboxylic

The effect of copper. In the early stages of this work we were puzzled by the occurrence of a pink band, running faster than, but not separated from, the main purple band. This was easily noticeable in the faster moving acids when benzyl alcohol, *tert.*-amyl alcohol, phenol, the cresols, *n*-butanol and

§ Cystine is decomposed by these additions. R_F value refers to the decomposition product.
|| Colour initially green. Only final colour shown in table.

quinoline were used as solvents. These 'pink fronts' were, however, absent when collidine and *iso*butyric acid were used. It was found that Cu in the paper was responsible for this effect. Thus, impregnation with $CuSO_4$ solution or rubbing the paper with metallic Cu increased the 'pink front' at the expense of the main band. The Cu salt of glycine ran as an extended pink band (suggesting adsorption on the paper) with no purple glycine band, and with an R_F value corresponding to that of the 'pink front' of glycine. Further, the 'pink front' material was converted back to glycine by H_2S, HCN and NH_3. Neither acid nor NH_3 washing of the paper removed all the Cu, which was present also in commercial acid-washed paper. It was found that reagents which precipitate or form complexes with Cu prevented 'pink front' formation, e.g. H_2S, HCN, NH_3, or coal gas in the atmosphere, or pretreatment of paper with $K_4Fe(CN)_6$, or the addition of a freshly prepared solution of 'cupron' (α-benzoinoxime) to the solvent.

When phenol is used in an atmosphere containing NH_3, the Cu present in the paper catalyzes the formation of fast-running black material (Kohn & Fryer, 1893), which is liable to spoil the lower part of the chromatogram. However, those reagents which prevent 'pink front' formation will also prevent this catalysis. Since some ammonium salts give colours with ninhydrin, the use of H_2S with NH_3 is precluded. A dilute solution of 'cupron' in the phenol (0·1 %), or a trace of HCN, produced by the addition of a few crystals of KCN to the tray, have been found convenient methods. Coal gas, to be satisfactory, must be used in high concentrations.

DISCUSSION

The reproducibility of R_F values depends on the constancy of the following six factors: paper, temperature, quantity of amino-acid, extraneous substances, degree of saturation with water, supply of solvent and distance between starting-point and source of solvent. The irregularity of the band across the width of a wide paper affords a measure of the variations of the R_F values due to inhomogeneity of the paper. Thus for the valine band in phenol on Whatman no. 1 paper, a variation of ± 2 % has been found. By measuring from the 'centre of gravity' of a band or spot, this variation is largely eliminated. Papers of different densities will give widely different R_F values. Thus Whatman no. 42 gives much slower rates than Whatman no. 1. The chief cause of variation with temperature has already been discussed. With the solvents tested, changes in temperature do not cause large changes in rates except with collidine. The quantity of amino-acid has little effect on the R_F value though the size of the spot varies considerably. The rates of some of the amino-acids are seriously affected by acids and by reagents used to suppress the effect of Cu in the paper. The rate of a given amino-acid is slightly altered by the presence of another amino-acid. The importance of maintaining a fully saturated atmosphere was not at first appreciated; in Table 3 the scatter is partly due to an ill-fitting lid on the chamber. The paper may be undersaturated or oversaturated (waterlogged), according to the state of the atmosphere (cf. Table 1). Unlike a chromatogram in a tube, the ratio of solvent to stationary phase varies at different levels in a strip of paper. This ratio becomes progressively greater the shorter the distance from the trough. This characteristic distribution is disturbed both by an interruption of the flow of liquid and by the liquid accumulating at the bottom of the strip. In both cases, the relative rates of the amino-acids are slightly modified. As a result of the distribution of solvent, the greater the distance between the trough and starting-point of the amino-acid, the smaller the R_F value. However, this effect may be neglected for normal working distances.

When strict duplicates are run simultaneously in the same chamber, differences in R_F value do not exceed 4 %. Runs done at long intervals of time differ by much more than this, since close control of all the relevant factors has not been attempted, but relative rates are much more constant. Hence while it is not always possible to identify a given amino-acid by its R_F value alone, comparison with other amino-acids on the same strip allows this to be done with confidence; e.g. where a protein hydrolysate has been run with a benzyl alcohol-*n*-butanol mixture (1:1 by vol.) it is possible to recognize phenylalanine, leucine, *iso*leucine and methionine. Of the six factors discussed above, only the presence of extraneous substances will alter the order of amino-acid bands on the strip. In the case of two-dimensional chromatograms, even the presence of acid or base, while modifying the pattern, does not prevent the recognition of the amino-acids. Colour, of course, serves for the identification of several of the acids.

Resolution of two amino-acids becomes difficult if the R_F values do not differ by more than about 10 %. Thus leucine and *iso*leucine (R_F values 1·13:1) are not completely separable in benzyl alcohol.

In deciding which pair of solvents to use on a two-dimensional chromatogram, the R_F values of the amino-acids in one solvent are plotted as ordinates against those in the other solvent as abscissae. Fig. 5 shows the collidine and phenol-ammonia diagram. This is to be compared with the photographs (Pls. 1, 2) of the chromatograms of a wool hydrolysate and a mixture of the 22 amino-acids. The diagram is plotted for equal travel of the solvents but, in the sheets photographed, the colli-

dine has been allowed to travel much farther than the phenol in order to make full use of the size of the sheets.

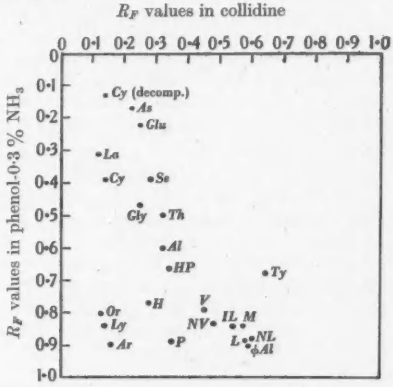

Fig. 5. Phenol-ammonia (0·3 %)/collidine diagram, showing expected positions of the amino-acids on a two-dimensional chromatogram prepared with these solvents. *Al*, alanine; *Ar*, arginine; *As*, aspartic acid; *Cy*, cystine; *Glu*, glutamic acid; *Gly*, glycine; *H*, histidine; *HP*, hydroxy-proline; *IL*, *iso*leucine; *La*, lanthionine; *L*, leucine; *Ly*, lysine; *M*, methionine; *NL*, *nor*leucine; *NV*, *nor*-valine; *Or*, ornithine; *φAl*, phenylalanine; *P*, proline; *Se*, serine; *Th*, threonine; *Tr*, tryptophan; *Ty*, tyrosine; *V*, valine.

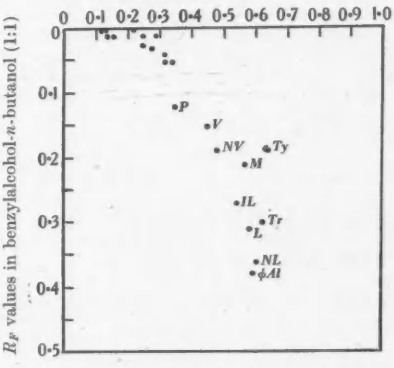

Fig. 6. Benzyl alcohol-*n*-butanol/collidine diagram, showing expected positions of the amino-acids on a two-dimensional chromatogram prepared with these solvents.

On the two-dimensional chromatogram, whatever the solvents, glycine and its straight chain homologues lie on a smooth curve. A branched chain results in slight deviation from this line, and a slowing of the rate. Since the presence of a hydroxyl group greatly decreases the R_F values in phenol, and has little effect on them in collidine, serine, threonine, tyrosine and hydroxyproline occur almost vertically above the corresponding unsubstituted amino-acids. Conversely, the bases are relatively slower in collidine than in phenol and thus occupy a position on the left of the diagram. It is of interest that proline, which has the same number of carbon atoms as valine, moves as fast as leucine in phenol and little faster than alanine in collidine; this behaviour is reminiscent of acetylproline, studied by Gordon *et al.* (1943*a*), which moved with acetylvaline or acetylalanine according to the solvent employed.

In Fig. 5, *iso*leucine, leucine, methionine and phenylalanine are crowded together, but by substituting *n*-butanol-benzyl alcohol mixture (1:1 v/v) for the phenol, a satisfactory separation of these components is obtained (see Fig. 6).

The small quantities of material employed by the method presented here are advantageous for many applications but make quantitative analysis difficult and preparative work impractical. Larger quantities can be handled in columns made from Whatman's 'Accelerator' paper, which can readily be reduced to powder, though advantage cannot then be taken of two-dimensional separation. Separations can be followed by periodic testing of a drop of effluent; material left in the column at any stage can be revealed by allowing a freshly prepared solution of ninhydrin (0·1 % in ether) to follow the solvent down the column. Coloured bands appear in a few hours at room temperature. For the weight of material handled these columns are bulky compared with those of silica gel. They have not as yet been investigated in detail but it is hoped that they will prove to be valuable. Columns of potato starch as used by Synge (1944) may prove to be superior.

The quantities of material employed in the two-dimensional technique are of the same order as those used in microbiological testing. Dr Happold kindly provided us with an opportunity of chromatographing a liver fraction containing growth factors for *C. diphtheriae*. Using a paper strip with *n*-butanol as solvent, at least six substances were shown to be present by treatment with ninhydrin. As in the case of the silica gel partition chromatograms, the paper chromatograms are by no means limited to the separations of amino-acids. Many bases and the NH_4 salts of many organic acids give colours with ninhydrin, and numerous colour reactions are available for other classes of compounds. The strip method can be employed to show the presence of more than one amino-acid in a given sample thought to be pure. The identity of a few micrograms of amino-acid can be established by running mixtures with authentic samples in a variety of solvents.

Any chromatographic method is well adapted for the discovery of further members of a known homo-

logous series, but the two-dimensional chromatogram is especially convenient, in that it shows at a glance information that can be gained otherwise only as the result of numerous experiments. The position of unknown substances on chromatograms developed with suitable pairs of solvents will also provide a clue to the existence of certain groups in the molecules, e.g. hydroxyl, acidic, basic or cyclic.

A few peptides have been shown to give satisfactory bands and also satisfactory colours with ninhydrin. In fact the work reported here was undertaken as a preliminary to the study of the products of the partial hydrolysis of proteins.

SUMMARY

1. A method of separating amino-acids on partition chromatograms by the use of water in cellulose (filter paper) as the stationary phase is described. Ninhydrin is used to reveal the amino-acids.

2. Phenol, collidine and *n*-butanol benzyl alcohol mixture (1:1 v/v) have proved useful as mobile phases. Other solvents have been investigated.

3. The partition coefficients calculated, normal water content of the paper being assumed, are close to those directly measured, showing that the cellulose acts as an inert support.

4. Two-dimensional chromatograms on sheets of filter paper are described; first one solvent is run in one direction, then, after the paper has been dried, another solvent is run in a direction at right angles to the first.

5. The presence of most of the amino-acids in wool, or in an artificial mixture of 22 amino-acids, can be demonstrated in a single experiment; all can be shown by suitable additional experiments. 200–400 μg. of protein are sufficient.

6. Hydroxy-amino-acids move more slowly than the corresponding amino-acids in phenol, but in collidine the rates are similar.

7. Ammonia selectively slows aspartic and glutamic acids and hastens the basic amino-acids. Acid has the reverse effect.

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Studies on Bacterial Amino-acid Decarboxylases

1. *l*(+)-LYSINE DECARBOXYLASE

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Amino-acid decarboxylases are produced by certain bacteria in response to growth in an acid environment (Gale, 1940, 1941), and it has been suggested that the neutralizing effect of the decarboxylation serves to protect the cell against the external acidity (Gale & Epps, 1942). The distribution of the decarboxylases among species and strains would indicate that each activity is produced by an enzyme specific for one amino-acid. The present communication deals with the extraction from the cell of *l*(+)-lysine decarboxylase, its partial purification, the properties of the partially purified enzyme and its resolution into the specific protein and a hitherto undescribed coenzyme. *l*(+)-Lysine decarboxylase is a component of the potential enzyme constitution of many of the coliform organisms, and two such organisms have been used as a source of enzyme in these studies. A preliminary note on the preparation of the specific enzyme and coenzyme has been published (Gale & Epps, 1943).

ORGANISMS USED

1. *Escherichia coli.* National Collection of Type Cultures no. 86.

2. *Bact. cadaveris.* This organism was isolated from pieces of meat left to putrify in phthalate buffer at pH 4. The organism was obtained by plating on to casein-digest-glucose-agar; we are indebted to Dr Tomlinson of the Emergency Public Health Service for the following characterization tests: acid and gas (24 hr.) in glucose, maltose, mannose; (5 days) in sucrose; no fermentation in lactose or salicin; indol negative; non-motile Gram-negative rod: the organism may therefore be classified in the paracolon group. A culture has been deposited with the National Collection under the classification no. 6578.

METHODS

Measurement of activity. The decarboxylation of *l*(+)-lysine was followed manometrically. The main cup of the Warburg manometer contained 2 ml. M/5 phosphate buffer pH 6·0 and 0·5 ml. enzyme preparation, with 0·5 ml. M/15 *l*(+)-lysine dihydrochloride (Roche Products Ltd.), neutralized to pH 6·0, in the side-bulb. The manometers were shaken in a bath at 30°, and after 15 min. equilibration the substrate was tipped in from the side-bulb. The CO_2 evolution was measured for the 15 min. after tipping. One unit of enzyme is defined as the amount which liberates 100 μl. CO_2 from *l*(+)-lysine in 5 min. at pH 6·0 and 30°. The purity of the preparations was followed by estimation of carbon content by the wet combustion method of Van Slyke & Folch (1940) and the activity expressed as $Q^{C}_{CO_2} = \mu$l. CO_2 liberated from *l*(+)-lysine at pH 6·0 and 30°/hr./mg. C of enzyme preparation.

Growth conditions for optimum production of enzyme. Previous work on washed suspensions of intact organisms has shown that the decarboxylase activity of coliform organisms is greatest when growth occurs in an acid medium and at a low temperature (Gale, 1940). These findings may be due to variation of enzyme content or alteration of the permeability of the cell membrane with growth conditions. Since the lysine decarboxylase activity of the cells is unaffected by acetone drying, it is possible to investigate this point with acetone powders of the organisms grown under varying conditions. Table 1, A shows the effect of the pH of the growth medium on the lysine decarboxylase activity of washed suspensions and of acetone-dried organisms: in either case the activity increases with decreasing growth pH so that there is an actual increase of enzyme in the cell in response to acid growth conditions. Highest activities are obtained when growth occurs in a casein-digest medium initially adjusted to pH 5·0, but this is near the acid limit of tolerance and yields are small; the presence of 2% glucose in the medium greatly increases the crop and provides, by the acidity of fermentation, organisms of a satisfactory activity level. Table 1, B shows the effect of growth temperature: low temperatures give rise to increased formation of enzyme; the highest activity was given by organisms grown at room temperature (*c.* 20°), but as this is too variable for satisfactory work, other cultures have been grown at 25°. A third factor of importance in growth conditions became apparent when we began to study the extraction of the enzyme from the acetone-dried organisms (as below): at first we found that our extractions varied from 10 to 75% of the total enzyme present from culture to culture. One of the causes of this variation appears to lie in the age of the culture at harvesting and Table 1, C shows that satisfactory extraction is obtained only from cells which have been grown under standard conditions for longer than 22 hr. It has been shown before (Gale, 1940) that cultures harvested early in the growth phase have little activity, but here we find that the enzyme, although developed, cannot be extracted satisfactorily from the dried cells unless growth is allowed to continue for about 24 hr. The standard conditions of growth adopted as a result of these findings consist of 24 hr. growth at 25° in 2% glucose-casein-tryptic-digest medium. To prepare a batch of enzyme we normally culture 15 l. of medium under these conditions, centrifuge down the organisms in a Sharples supercentrifuge, wash the cells once with water and make them into a thick cream with water ready for treatment with acetone.

Table 1. *Optimal growth conditions for the production and extraction of* l(+)*-lysine decarboxylase* (Esch. coli)

	Q_{CO_2} (lysine)	
A. *pH of medium* (during growth at 27°)	Washed suspension	Acetone powder
7·0	118	218
6·0	250	426
5·0	410	721
2% glucose broth (final pH = 5·2)	254	430
B. *Growth temperature* (in 2% glucose broth)		
37°	55	156
27°	205	337
Room temp. (*c.* 20°)	252	421

C. *Effect of age of culture on extractability*

Age of culture at 25° in 2% glucose broth (hr.)	Suspension of acetone powder (units/ml.)	Supernatant (units/ml. extract)	Extraction (%)
14	2·82	0·48	17
18	3·16	1·26	40
22	3·10	2·04	66

PRELIMINARY STUDIES ON THE PREPARATION OF THE ENZYME

Preparation of acetone powder and extraction of enzyme therefrom

The thick cream of washed organisms is poured rapidly into 5 vol. acetone and then stirred vigorously until coagulation occurs. The coagulum is allowed to settle and the supernatant liquid, which should be nearly or quite clear, is poured off. The rest of the material is filtered on a Büchner funnel (capacity *c.* 100 ml.) with slight suction, the filtrate being passed through again if necessary. When the cake is almost dry and beginning to crack, it is flooded with acetone and the whole worked up to a cream before suction is again applied. When the

acetone has been removed, this operation is repeated with *c.* 100 ml. dry ether. Suction is maintained until the powder is dry, when it is crumbled and spread out on a glass dish in the air to dry. Powders prepared in this way show an apparent increase in lysine decarboxylase activity, estimated on a carbon basis (Table 1), and retain this undiminished for a long period; but preparations from *Esch. coli* are best preserved by drying and storing *in vacuo*.

Preliminary experiments indicated that it is possible to extract the lysine decarboxylase from the acetone-powdered organism by incubation with buffer. Table 2 shows the degree of extraction obtained in 2 hr. at 27° with various buffer solutions: in each case 70 mg. of the powder were suspended in 2·5 ml. buffer in a centrifuge tube, the suspension incubated for 2 hr. at 27° and the sediment then spun off; 0·5 ml. of the clear supernatant was taken for test at pH 6·0. Table 2 shows that extraction is favoured by mildly alkaline conditions, being optimal between pH 8·0 and 9·0. Investigation of the effect of time and temperature on the extraction by M/45 borate buffer pH 8·5 shows that the process is rather more efficient at 37°, and for further experiments the powder was extracted for 2 hr. at 37° in M/45 borate buffer pH 8·5, with 1 ml. buffer/20 mg. powder.

Table 2. *Variation of extraction of enzyme with* pH

(Contents of tubes: 70 mg. acetone powder + 2·5 ml. buffer. Incubated for 2 hr. at 27° and sediment centrifuged off; test carried out at pH 6·0 on 0·5 ml. clear supernatant in each case.)

Buffer	pH	Activity of extract (μl. CO_2/0·5 ml./10 min.)
M/45 phthalate	4·0	4
	5·0	23
M/45 phosphate	6·0	139
	7·0	141
M/45 borate	8·0	220
	9·0	202
	10·0	34
	11·0	14

It has been shown above that extraction takes place more readily from cells grown for 22–24 hr. but there are other, as yet unknown, factors controlling the ease of extraction from the acetone powder and considerable variations are encountered from preparation to preparation made under apparently identical conditions. If the percentage extraction is less than 20, a further yield can sometimes be obtained by repeating the extraction process.

Preliminary experiments on the purification of the extracted enzyme

Acidification. The extract obtained in this manner is an opalescent, faintly yellow solution. On acidification a precipitate is formed which increases in bulk as the pH falls. Attempts were made to effect a purification in this way, but it was found that precipitation in buffers of pH below 5·0 resulted in complete loss of activity.

Adsorption on to and elution from alumina C_γ. Aliquots of the crude enzyme extract were adjusted by means of buffer solutions to pH 5–10, and an equal volume of alumina C_γ suspension (16 mg./ml.; pH 7·0) added. After standing for 10 min., the alumina was centrifuged off and the activity of the supernatant tested. It was found that the enzyme is adsorbed at mildly acid or alkaline values and that the enzyme is active in the adsorbed state on the alumina. Accordingly a more accurate estimate of the amount adsorbed can be made by suspending the alumina in the initial volume of water and testing the activity of this suspension. Table 3, A, shows the results of one such experiment, in which optimal adsorption occurred when the extract was adjusted to pH 6·0 before the addition of the alumina C_γ. A further experiment was carried out in which the proportion of alumina suspension to extract was varied, after preliminary adjustment to pH 6·0. Table 3, B, shows the amount of activity

Table 3. *Adsorption of the enzyme on alumina* C_γ

A. *Optimum* pH *for adsorption*

(1 ml. crude extract + 1 ml. buffer + 1 ml. alumina C_γ suspension: left standing 10 min. and alumina centrifuged off; alumina resuspended in 3 ml. phosphate at pH 6·0 and 1 ml. taken for test.)

pH during adsorption	Activity of suspension (μl. CO_2/ml./5 min.)
5·0	90
6·0	135
7·0	32
8·0	129
9·0	114
10·0	29
Total activity before centrifuging	151

B. *Proportion of alumina suspension*

(Tubes set up with 1 ml. crude enzyme extract adjusted to pH 6·0. Various amounts of alumina C_γ suspension (16 mg./ml.) added and volume made up to 2 ml. with water; after 10 min. alumina centrifuged off and 0·5 ml. supernatant taken for test at pH 6·0.)

Alumina C_γ suspension added (ml.)	Activity of supernatant (μl. CO_2/ml./5 min.)
0·0	189
0·1	127
0·2	48
0·4	19
0·6	17
0·8	11

left in the supernatant after the alumina has been removed in such an experiment; most of the enzyme is adsorbed if the acidified extract is treated with half its volume of standard alumina C_γ suspension. Elution was tested with phosphate buffers at various pH levels and was found to be complete in M/5 phosphate buffer at pH 7·0: on a larger scale, elution is not always complete in one operation and it has been the custom to elute twice, each elution being made with a volume of M/5 phosphate pH 7·0 equal to that of alumina suspension originally used for adsorption.

Separation of decarboxylases. Washed suspensions of *Esch. coli* grown and prepared as above contain the decarboxylases of arginine, lysine, histidine and ornithine. The ornithine enzyme is destroyed by the acetone-drying process, whereas the other three enzymes are unaffected. These latter have very similar properties, and the extraction process, adsorption with, and elution from alumina C_γ take place under the same conditions for all three; hence the procedures described above do not bring about any separation of the three enzymes. During the early investigations on the possibility of acid precipitation of the crude extract, it appeared that the lysine enzyme was rather more stable to mild acidity than those of arginine and histidine. Accordingly, attempts were made to effect a separation of the enzymes by acid precipitation in the presence of ethanol. After various trials it was found that if the pH of the crude extract is adjusted to 5·5 or 6·0 in the presence of 20% ethanol, a precipitate is formed which contains the bulk of the lysine decarboxylase activity but little arginine activity (*vide* Table 4, A). It is important that the ethanol be added before and not after acidification, otherwise general loss of activity occurs. The histidine enzyme is destroyed to the same extent as the arginine decarboxylase by this procedure.

It was found that the lysine decarboxylase activity can be stabilized under these conditions if adsorption on to alumina C_γ is combined with the precipitation by acid in the presence of 20% ethanol. Table 4, B, shows that if the crude extract is adjusted to pH 6·0 with 3% acetic acid in the presence of 20% ethanol, and one-half volume of alumina C_γ suspension is then added, the supernatant left after removal of the alumina contains little lysine or arginine activity, while a suspension of the alumina in the original volume of water carries the greater part of the lysine enzyme in an adsorbed state. Elution of the alumina with M/5 phosphate at pH 7·0 results in a preparation very active with respect to lysine and containing little arginine enzyme.

Table 4. *Acid-ethanol precipitation and adsorption on to alumina* C_γ

	Activity (μl. CO_2/0·5 ml./5 min.) with substrate	
A. Preparation process	Arginine	Lysine
1. Crude enzyme extract	30	137
2. Adjust to pH 5·5 with acetic acid; add ethanol to 20%; remove and dissolve precipitate in original volume	4	8
3. Add ethanol to 15%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	6	89
4. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	2	99
B. 1. Crude enzyme extract	34	204
2. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	6	169
3. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; add half vol. alumina C_γ suspension; leave 10 min.; remove alumina; test supernatant	5	6
4. Suspend alumina from (3) in original volume of water	3	136
5. Eluate in M/5 phosphate from alumina (4)	2	131
6. Eluate corresponding to (5) from alumina on which adsorption occurred in absence of ethanol	26	156

Bact. cadaveris possesses a very active lysine decarboxylase, a very weak arginine decarboxylase and no other enzymes of this group. When the above procedure is followed in the case of extracts prepared from acetone-dried *Bact. cadaveris*, the arginine enzyme is completely destroyed and the eluate is specific for lysine.

Adsorption will occur on to calcium phosphate under much the same conditions as those discussed for alumina C_γ, but elution is more difficult and will only occur satisfactorily in the presence of 15% ammonium sulphate. It will be shown below that the presence in the preparations of ammonium sulphate has an inhibitory effect and consequently the use of calcium phosphate as an adsorbent was discarded.

PREPARATION OF *l*(+)-LYSINE DECARBOXYLASE

Table 5 shows the steps in the purification of lysine decarboxylase worked out on the basis of the preliminary findings described above. As each step of

Table 5. *Purification of* l(+)*-lysine decarboxylase from* Esch. coli *and* Bact. cadaveris

Stage	Preparation	Source of enzyme ...	*Esch. coli*					*Bact. cadaveris*				
			ml.	Activity/ml.	Units	mg. C/ml.	$Q^{C}_{CO_2}$	ml.	Activity/ml.	Units	mg. C/ml.	$Q^{C}_{CO_2}$
1	Intact organism suspension		—	196	—	4·12	508	—	—	—	—	963
2	Acetone powder suspension		230	744	1610	10·4	860	90	1100	990	7·2	1,830
3	Crude extract		225	455	1022	2·95	1,850	84	1100	924	6·1	2,170
4	Combined eluates from alumina C_γ		204	348	710	1·02	4,100	84	885	745	—	—
5	Protein precipitated with 50 g. $(NH_4)_2SO_4$/100 ml.		100	571	571	1·63	4,210	54	1125	607	3·28	4,500
6	First $(NH_4)_2SO_4$ fraction:											
	(*a*) 0–40% saturated $(NH_4)_2SO_4$		25	170	42	1·50	1,360	20	500	100	1·74	3,450
	(*b*) 40–56% saturated $(NH_4)_2SO_4$		25	1700	420	0·97	21,100	25	1890	472	2·97	7,650
	(*c*) 56–66% saturated $(NH_4)_2SO_4$		25	111	23	1·15	1,160	20	90	18	—	—
7	Second fractionation of 6 *b*:											
	(*a*) 0–40% saturated $(NH_4)_2SO_4$		15	880	132	0·869	12,200	15	475	71	—	—
	(*b*) 40–47% saturated $(NH_4)_2SO_4$		15	1540	232	0·448	41,300	15	2600	390	1·78	17,500
	(*c*) 47–52% saturated $(NH_4)_2SO_4$		15	112	17	0·353	3,780	15	20	3	—	—
8	Third fractionation of 7 *b*:											
	(*a*) 0–41% saturated $(NH_4)_2SO_4$		10	Negligible	—	—	—	10	370	37	—	—
	(*b*) 41–47% saturated $(NH_4)_2SO_4$		*10*	*800*	*80*	*0·209*	*46,000*	*10*	*3120*	*312*	*1·12*	*33,500*
	(*c*) 47–52% saturated $(NH_4)_2SO_4$		10	1310	131	0·49	32,000	10	92	9	—	—
9	Fraction 8 *b* adsorbed on to and eluted from alumina C_γ		7	578	41	0·269	25,700	—	—	—	—	—

the purification was carried out, the following measurements were made: volume of dissolved preparation, the lysine decarboxylase activity of a suitable measured fraction, expressed as μl. CO_2 liberated from *l*(+)-lysine/5 min./ml. preparation, and the C content of a suitable fraction. From these values the number of enzyme units and $Q^{C}_{CO_2}$ were calculated as a measure of the yield of material and degree of purification respectively. The table shows figures obtained for preparations starting from washed suspensions of both *Esch. coli* and *Bact. cadaveris*. The stages in the preparation are as follows.

1. Thick washed suspension of organism grown for 24 hr. at 25° in 15 l. 2% glucose-casein-tryptic-digest broth.

2. The washed suspension is treated with 5 vol. acetone, filtered off on a Büchner funnel, washed with acetone and then with ether, and dried in air. The dry powder is broken up, weighed and suspended in M/45 borate buffer, pH 8·5, using 1 ml. buffer/20 mg. powder.

3. The suspension of acetone powder in buffer is incubated, with occasional stirring, for 2 hr. at 37°. The suspension is then centrifuged at 3000 r.p.m. for 15 min. to remove the inactive sediment. At this stage the degree of extraction should be tested. Generally good yields and clean fractionation are obtained in the later stages of the preparation only if the extraction exceeds 50% at this stage.

4. Ethanol is added to a final concentration of 20%, and 3% acetic acid then added with stirring until the pH lies between 5·5 and 6·0 (yellow to bromothymol-blue; blue to bromocresol-green). A volume of alumina C_γ suspension (16–20 mg./ml.), equal to half the volume of extract at stage 3, is then added and the whole left to stand for 10 min. The alumina is centrifuged and the supernatant discarded. The alumina is resuspended in M/5 phosphate, pH 7·0, of the same volume as the amount of alumina C_γ suspension taken for adsorption. After 10 min. the alumina is again centrifuged, the supernatant collected, and the elution repeated under the same conditions. The eluates are combined. Yields over this stage vary between 70 and 80%, with a purification of 200–250%.

5. The volume of the combined eluates is measured and 50 g. solid ammonium sulphate added/100 ml. The ammonium sulphate is stirred in until dissolved and the solution left to stand for 10 min. The precipitate is then centrifuged for *c.* 30 min. at 3000 r.p.m. The supernatant solution is discarded, the precipitate drained as far as possible and then dissolved in a convenient amount of water. There is usually a small loss of material at this stage, but the further fractionation is more constant and precipitates centrifuge more easily after this bulk precipitation. On solution in water there is usually an insoluble residue which can be removed by centrifuging if the material is to be used at this stage without further purification. The preparation from *Bact. cadaveris* is specific for lysine at this stage and can be used for the estimation of lysine, protein analysis, etc.

6. Further purification is carried out by progressive ammonium sulphate fractionations. The volume of the preparation, stage 5, is measured and sufficient saturated ammonium sulphate solution is added to bring the final concentration to 40 % saturation. After standing 5 min., the precipitate is centrifuged off, 15 min. at 3000 r.p.m. being sufficient to pack the material in the tube. The ammonium sulphate concentration in the supernatant is then raised to 56 % by the addition of the calculated quantity of saturated solution and the second fraction centrifuged down separately. The third fraction is precipitated by raising the concentration to 66 % saturation. Each fraction is dissolved in water for test, and the bulk of the enzyme is found in the middle fraction.

7. The middle fraction is now refractionated between the limits indicated in Table 5 and most of the activity comes out in the 40–47 % saturated ammonium sulphate fraction, effecting a purification of 2–3 times over stage 6.

8. The middle fraction from stage 7 is again refractionated over approximately the same limits. With the *Bact. cadaveris* preparation this third fractionation results in the removal of more inactive protein, giving a purification of 200 % in the middle fraction, which still separates cleanly. The material from *Esch. coli* begins to be divided among the fractions at this stage and although most of the enzyme comes down in the 47–52 % fraction, the highest activity is still shown by the 41–47 % range. The arginine activity remaining in preparations obtained from *Esch. coli* after stage 4 accompanies the lysine activity until this stage, when separation occurs, most of the arginine activity appearing in the 47–52 % fraction. The 41–47 % fraction always contains some arginine activity when *Esch. coli* is the source, and the $Q^{C}_{CO_2}$ (arginine) lies between 500 and 1500.

9. Further ammonium sulphate fractionation after stage 8 results in division of the enzyme among the fractions, without significant purification. In an attempt to remove the remaining traces of arginine activity from the *Esch. coli* preparation, adsorption on to alumina at pH 6·0 in the presence of 20 % ethanol followed by elution in phosphate was tried again with fraction 8 *b*. This procedure resulted, however, in a loss of activity without significant separation of the enzymes.

For the investigation of the properties of the enzyme, material obtained in fraction 8 *b* has been used, with *Bact. cadaveris* as the source. In a small percentage of preparations a trace of arginine decarboxylase activity survives destruction, but this disappears if the preparation is kept for 3–4 days in the ice-chest. Preparations made from *Bact. cadaveris* and dissolved in M/200 phosphate pH 6·0 can be kept in the ice-chest for 2–3 weeks without serious deterioration. Preparations from *Esch. coli* lose up to 50 % of their activity within 24 hr., partly due to spontaneous resolution of the protein-coenzyme complex (see later). The preparations form clear colourless solutions in water.

A preparation from *Esch. coli*, fraction 8 *b*, was centrifuged on the ultra-centrifuge for us by Dr A. G. Ogston at the Biochemical Department, Oxford. The material was non-homogeneous.

Ultra-violet absorption spectrum. The preparations give typical protein ultra-violet absorption spectra with a peak at 265 mμ. The preparation 8 *b*, $Q^{C}_{CO_2} = 33{,}500$, source *Bact. cadaveris*, gave extinction $\log_{10} I_0/I = 1{\cdot}5$ at 265 mμ ($d = 2$ cm.) for a solution containing 0·28 mg. C/ml.

PROPERTIES OF THE PURIFIED ENZYME

pH *optimum*. Fig. 1 shows the variation of *l*(+)-lysine decarboxylase activity with the reaction pH. The optimum lies at pH 6·0, whether phthalate, phosphate or citrate buffers are used. In most experiments reported, the buffer used was M/5 phosphate, but M/5 citrate buffer was used for the investigation of certain metal inhibitors. The

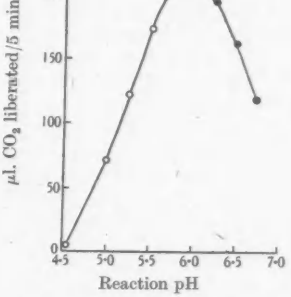

Fig. 1. Activity-pH curve for *l*(+)-lysine decarboxylase. o—o M/10 phthalate buffers. •—• M/5 phosphate buffers. Contents of cups: 0·5 ml. enzyme; 2·0 ml. buffer; 0·5 ml. M/15 *l*-lysine. Temp. 30°.

pH optimum of the purified enzyme differs from that measured with the intact organism, which may lie anywhere between 4·5 and 5·0, varying from culture to culture, the curve showing an abrupt drop on the acid side of the optimum (Gale, 1940). We have suggested that this difference in the pH optimum measured with the intact cell and with the cell-free enzyme is due to the pH difference between the internal and external environments of the cell produced as a result of the neutralizing effect of the decarboxylase action (Gale & Epps, 1942).

Effect of substrate concentration. Fig. 2 shows the variation of velocity of decarboxylation with concentration of substrate for the enzyme preparations from the two sources. The two curves are very similar, though it would appear that the enzyme

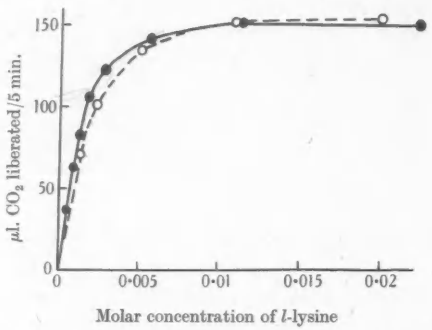

Fig. 2. Effect of lysine concentration on lysine decarboxylase activity. •—• Enzyme from *Bact. cadaveris.* o- - -o Enzyme from *Esch. coli.* Contents of cups: 0·5 ml. enzyme; 2·0 ml. M/5 phosphate pH 6·0; 0·5 ml. substrate solution. Temp. 30°.

from *Bact. cadaveris* has a slightly higher affinity for the substrate than that isolated from *Esch. coli.* In both cases the value of the Michaelis constant falls between 0·0012 and 0·0018M. In our experimental tests, the final concentration of substrate initially present in the manometer cups is M/90, which is sufficient to saturate the enzyme from either source.

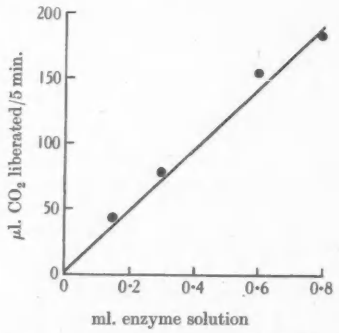

Fig. 3. Proportionality between CO_2 liberation and enzyme concentration.

Effect of enzyme concentration. Fig. 3 shows that a linear relation exists between velocity of reaction and enzyme concentration over the range used in manometric tests.

Effect of salt concentration. In the course of preliminary work, we noticed that the presence of ammonium sulphate in our preparations appeared to have an inhibitory effect. Fig. 4 shows the effect on the velocity, in M/5 phosphate pH 6·0, of the presence of various concentrations of ammonium sulphate (curve 2): concentrations of the order 0·05M (1 % saturated ammonium sulphate) have a significant effect. Similar results are obtained with Na_2SO_4 or NaCl. Since dialysis of the preparations to remove salts results in irreversible inactivation of the enzyme, methods of elution from adsorbents, etc., involving the use of salts have been avoided.

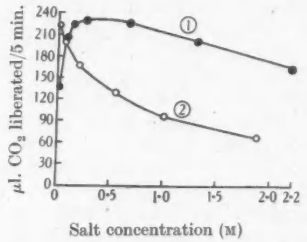

Fig. 4. Effect of salt concentration on lysine decarboxylase activity. (1) •—• Phosphate (pH 6·0). (2) o—o Ammonium sulphate in presence of 0·2 M phosphate pH 6·0.

Curve 1, Fig. 4, shows the effect of varying the concentration of phosphate buffer: there appears to be an optimum concentration of buffer corresponding to 0·1–0·5M. Below this concentration there is a loss of activity, probably due to inefficient buffering action, while the effect of high concentrations appears to be similar to that obtained with ammonium sulphate. Manometric tests have been carried out in 0·2M phosphate.

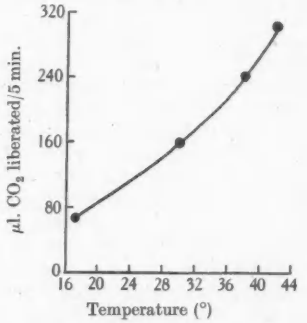

Fig. 5. Effect of temperature on activity of lysine decarboxylase.

Effect of temperature. Fig. 5 shows the variation of velocity with reaction temperature: the temperature coefficient over the range 30–40° is 1·7.

(Experimental tests were always made in a thermostat at 30° and all the rates quoted are for this temperature.)

Substrate specificity. Of the following 15 common amino-acids tested the only one attacked by the purified enzyme preparation (*Bact. cadaveris*) is *l*(+)-lysine: *l*(+)-lysine, *d*(−)-lysine, *l*(+)-arginine, *l*(+)-glutamic acid, *l*(+)-ornithine, *l*(−)-histidine, *dl*-alanine, *dl*-valine, *l*-leucine, *l*(−)-phenylalanine, *l*(−)-tyrosine, *l*(−)-tryptophan, *dl*-serine, *l*(−)-proline, *l*(−)-aspartic acid. The action on α-acetyl, α-methyl, ε-acetyl, ε-methyl-derivatives of lysine, prepared by Dr F. Sanger of this laboratory, was also tested: since no activity was obtained in any of these cases it would seem that the integrity of both α and ε amino-groups in the lysine molecule is necessary for decarboxylase activity. Piperidine-α-carboxylic acid was not attacked. The enzyme is optically specific and will not decarboxylate *d*(−)-lysine. Use has been made of this activity to resolve *dl*-lysine and prepare the unnatural isomer (Neuberger & Sanger, 1944).

Fig. 6 shows the course of decarboxylation of a sample of hydroxy-lysine kindly given to us by Dr D. D. Van Slyke. The sample contained about 13% lysine and the analytical data supplied by Dr Van Slyke were: NH_2-N, 14·24; C, 36·89; Cl, 18·34%; hydroxy-lysine, estimated from the ammonia evolved with periodate, 86·5%; $[\alpha]_D$ in

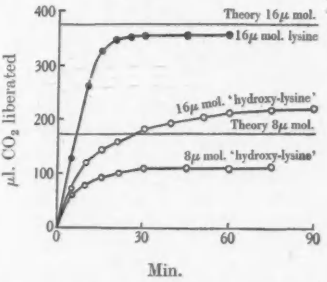

Fig. 6. Decarboxylation of *l*-lysine and hydroxy-lysine (13% lysine)

N-HCl, +4·7°. For test, 16μmol. *l*(+)-lysine, 16μmol. and 8μmol. hydroxy-lysine were shaken with the enzyme preparation. The decarboxylation of the lysine proceeded normally to 92% completion. An initial rapid evolution of CO_2 was obtained with the 'hydroxy-lysine' due to the lysine present in the preparation; this was followed by a slower evolution of CO_2, eventually ceasing at a quantity corresponding to the decarboxylation of approximately half the theoretical amount of hydroxy-lysine present. The CO_2 evolution corresponds to the decarboxylation of something in the preparation other than lysine. It is possible that the hydroxy-lysine is racemized during preparation and that only the natural isomer is attacked or, alternatively, that there may be more than one hydroxy-lysine. Further investigations were not possible owing to lack of material. We have found that tyrosine decarboxylase will decarboxylate dihydroxyphenylalanine (Epps, 1944) and that glutamic acid decarboxylase will attack synthetic β-hydroxyglutamic acid; therefore the introduction of an —OH group into the substrate molecule does not prevent the action of other decarboxylases, although it decreases the rate of decarboxylation in the three cases studied.

Quantitative nature of the decarboxylation of lysine. Using 0·5 ml. M/30 *l*(+)-lysine (Roche Products Ltd.) under the standard manometric conditions described above, a series of 19 tests gave mean CO_2 output = 344μl., standard deviation 4·7, range 337–354. Theoretical decarboxylation = 373μl. The experimental output under these conditions is thus 92% of calculated, but working at pH 6·0 there is some retention of CO_2, and if this is estimated at the end of the experiment by the addition of acid with a correction for blanks (Woods & Clifton, 1937), the mean gas output from 10 tests = 366μl., standard deviation 5·1, range 359–378; this represents 98% of calculated, so that the decarboxylation is quantitative.

If *dl*-lysine is used as substrate, the mean gas output from 0·5 ml. M/30 solution is 164μl., corresponding to 45% of calculated.

Identification of product. The following quantities were put up in an Erlenmeyer flask: 15 ml. M/30 *l*(+)-lysine, 60 ml. M/5 phosphate pH 6·0, and 7·5 ml. enzyme preparation (70 units lysine decarboxylase). The flask was shaken at 30° for 30 min. and the contents were then deproteinized with colloidal iron. The amine product was isolated as the picrate by the method previously described (Gale, 1940): yield 178 mg. Analysis of the product twice recrystallized from hot water (F. Weiler, Oxford): C, 36·29; H, 3·59; N, 19·75%. Calc. for cadaverine picrate: C, 36·42; H, 3·57; N, 20·0%.

Inhibitors. Table 6 lists the lowest molar concentrations of various common inhibitors which will produce 95–100% inhibition of lysine decarboxylase. The enzyme is very sensitive to silver and mercury, considerably less sensitive to copper and iron. Hydroxylamine and hydrazine are effective inhibitors in concentrations of 10^{-5} and 10^{-4} M respectively; 10^{-3} M-cyanide or azide produce 95% inhibition, which suggests the presence of a metal in the enzyme constitution. However, sodium sulphide, carbon monoxide and the 'copper inhibitors' (8-hydroxy-quinoline, sodium diethyl-dithio-carbamate and α-benzoin-oxime (Stotz, Harrer & King, 1937)) have no significant inhibitory action in

concentrations below 0·01 M. We are indebted to Dr Nicholson for spectrometric examination of ashed enzyme for metals: the only metals found were Ca, Ba and Sr, which were equally present in control portions of the ammonium sulphate used.

Table 6. *Inhibitors of* l(+)*-lysine decarboxylase*

(−log (*I*)=negative log of molar concentration of inhibitor.)

Inhibitor	−log (*I*)	Inhibition (%)
$HgCl_2$	5	100
$AgNO_3$	5	98
NH_2OH	5	100
$NH_2.NH_2$	4	95
$KMnO_4$	4	100
HCN	3	93
NaN_3	3	98
$CuSO_4$	3	89
$FeSO_4$	3	82
Na_2S	2	32
Iodoacetate	2	20
Sulphanilamide	2	15

Substances ineffective in concentrations below 0·01 M: NaF, urethane, chloramine T, 8-hydroxyquinoline, Na diethyl-dithio-carbamate, α-benzoin-oxime, sulphathiazole, carbon monoxide.

That the cyanide inhibition is reversible was demonstrated in the following way. Manometers were put up in duplicate containing final concentrations of 0·001 and 0·0001 M cyanide, together with controls without cyanide. In one of each pair, 0·3 ml. 40 % NaOH was placed in the centre cup, with a fluted filter paper strip dipping into the alkali. The manometers were then shaken in the bath at 30° with their taps closed for 1 hr. At the end of that time the filter papers were removed, the alkali cleaned out and the centre cups acidified. The manometers were then reassembled and, after the usual equilibration period, the substrate added in all manometers. Table 7 shows that cyanide exerts its usual inhibitory effect in those manometers without alkali, whereas the inhibitory effect has been reversed to a large extent in those manometers where alkali in the centre cup has absorbed part of the cyanide.

Table 7. *Reversibility of cyanide inhibition*

Manometer no. ...	1	2	3	4	5	6
Enzyme solution (ml.)	0·2	0·2	0·2	0·2	0·2	0·2
M/5 phosphate pH 6·0 (ml.)	2·0	2·0	2·0	2·0	2·0	2·0
M/15 l(+)-lysine (ml., in side-bulb)	0·3	0·3	0·3	0·3	0·3	—
6 M/1000 cyanide (ml.)	—	0·5	0·5	—	—	—
6 M/10,000 cyanide (ml.)	—	—	—	0·5	0·5	—
40% NaOH (ml., in centre cup)	—	—	0·3	—	0·3	—
Water (ml.)	0·5	—	—	—	—	0·8
Incubate 1 hr. at 30°; remove NaOH from nos. 3 and 5; equilibrate; tip in the substrate:						
Gas evolution (μl. CO_2/5 min.)	163	8	63	53	135	0
Inhibition (%)	—	95	61·5	69	17	—

REVERSIBLE RESOLUTION INTO SPECIFIC PROTEIN AND COENZYME

Dialysis of the purified enzyme preparations results in rapid and irreversible inactivation; preparations from *Esch. coli* are completely inactivated by 12 hr. dialysis against distilled water; preparations from *Bact. cadaveris* lose approx. 50 % of their activity in 24 hr. dialysis. Previous studies with washed suspensions of *Esch. coli* have shown that the lysine decarboxylase activity can be increased by the addition of a boiled suspension of yeast to the bacteria during test. These findings suggest the presence of a dialyzable coenzyme which could not, in the case of intact organisms, be replaced by aneurin diphosphate (Gale, 1940).

Table 8 shows that repeated precipitation of a lysine decarboxylase preparation (stage 7 *b*, source *Esch. coli*) with 66 % saturated ammonium sulphate at pH 9·0 results in the preparation of a protein with little activity towards lysine, but whose activity is considerably increased by the addition of boiled yeast suspension. Further work along these lines has shown that the following is the most satisfactory method of preparing the inactive apo-enzyme.

When *Esch. coli* is the source of the enzyme, the preparation is followed as described as far as stage 5. The precipitate obtained at this stage is dissolved in water and insoluble material centrifuged off. The enzyme is then precipitated once with 66 % saturated ammonium sulphate, dissolved in water and again precipitated with 66 % saturated ammonium sulphate solution containing 10 % ammonia. The precipitate does not centrifuge easily and requires 30 min. at 3000 r.p.m. to pack down. The precipitate is again dissolved and reprecipitated with ammoniacal ammonium sulphate as before; the resolution is aided if the material is left to stand for 15–20 min. before centrifuging. The precipitate is now dissolved in a suitable amount of M/5 phosphate pH 6·0 and tested with and without sufficient coenzyme (co-decarboxylase) to saturate the protein. Considerable loss of material usually accompanies this resolution, sometimes amounting to 60–70 % of the

enzyme. The activity towards lysine of the apo-enzyme preparation, in the absence of added coenzyme, is very small and can be still further reduced by a third alkaline precipitation if desired, although the resolution is best completed by allowing the preparation to stand in solution in the ice-chest for several days. The solution of apo-enzyme can be kept for several weeks in the ice-chest. Table 8 also shows the effect of adding boiled yeast suspension to apo-enzyme prepared in this way from enzymes isolated from *Esch. coli* and *Bact. cadaveris*. The resolution of the enzyme from *Esch. coli* is accomplished far more easily than that of the enzyme from *Bact. cadaveris* and this difference seems to be correlated with the greater stability on keeping and on dialysis of the latter preparation.

Table 8. *Reversible resolution by precipitation with alkaline 66 % saturated ammonium sulphate*

(Preparations made up to original volume after each precipitation.)

Enzyme preparation: lysine decarboxylase preparation stage 7 *b*; source, *Esch. coli*	Activity (μl. CO_2/0·5 ml./10 min.) Alone	+ boiled yeast	Increase due to yeast (%)
Precipitated once at pH 7·0	282	332	18
Precipitated once at pH 9·0	124	265	113
Precipitated twice at pH 9·0	81	204	152
Precipitated thrice at pH 9·0	35	198	465
Twice precipitated with ammoniacal ammonium sulphate as described in text:			
(*a*) source, *Esch. coli*	14	208	1385
(*b*) source, *Bact. cadaveris*	150	223	49

Boiled yeast suspension forms a useful source of coenzyme for general testing purposes but the apo-enzyme can also be reactivated by boiled suspensions of *Esch. coli* or *Bact. cadaveris*, or by boiled preparations of purified lysine decarboxylase itself.

By the use of the decarboxylation of lysine by apo-enzyme preparation in the presence and absence of various substances under the usual conditions, the following substances have been tested for coenzyme activity with negative results: coenzyme I, coenzyme II, aneurin, aneurin diphosphate, riboflavin-adenine-dinucleotide, flavin-mononucleotide, riboflavin, adenylic acid, adenosine, inosine, hypoxanthine, glutathione, yeast nucleic acid, yeast nucleic acid hydrolyzed with NH_4OH, nicotinic acid, nicotinic amide, uracil, pantothenic acid, biotin, pyridoxin, *p*-amino-benzoic acid, inositol, thiochrome: aneurin diphosphate + ashed yeast, or Mg, Ca, Zn, Fe, Cu, Mn, Co, etc.; aneurin diphosphate + riboflavin-adenine-dinucleotide or riboflavin-mononucleotide or riboflavin; aneurin or thiochrome + riboflavin-adenine-dinucleotide or riboflavin-mononucleotide or riboflavin.

The preparation of the codecarboxylase from brewer's yeast is described in a further paper of this series (Gale & Epps, 1944). Table 9 gives the results of an experiment designed to show the recombination of apo-enzyme and codecarboxylase.

To determine the half-saturation concentration of codecarboxylase, manometer cups were assembled with the following contents: 0·25 ml. apo-enzyme preparation equivalent to 2 units of enzyme, 1·75 ml. M/5 phosphate buffer pH 6·0, in the main part, and 0·5 ml. M/15 *l*(+)-lysine in the side-bulb. To a series of such cups was added 0·5 ml. phosphate buffer containing amounts of codecarboxylase corresponding to 0·01–2·5 μg. carbon, the preparation used being of the highest purity so far prepared (Gale & Epps, 1944). The manometers were equilibrated at

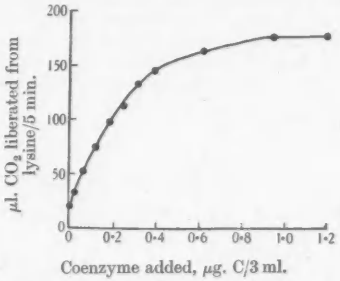

Fig. 7. Effect of coenzyme concentration on rate of decarboxylation of *l*-lysine by apo-enzyme. Contents of cups: 0·25 ml. apo-enzyme solution; 0·5 ml. M/15 *l*-lysine; 2·0 ml. M/5 phosphate buffer pH 6·0; 0·25 ml. Codecarboxylase solution as above. Temp. 30°.

30° for 15 min. before the substrate was added and the steady rate of decarboxylation was then studied for a further 15 min. Fig. 7 shows the dependence of the velocity of decarboxylation on the concentration of codecarboxylase present. Half-saturation of

Table 9. *Recombination of apo-enzyme and codecarboxylase*

Manometer no. ...	1	2	3	4	5	6
M/5 phosphate pH 6·0 (ml.)	1·75	1·75	1·75	1·75	1·75	1·75
Apo-carboxylase preparation (ml.)	0·25	0·25	0·25	0·25	—	—
M/15 *l*(+)-lysine (ml., in side-bulb)	0·5	0·5	—	—	0·5	—
Codecarboxylase preparation (ml.)	—	0·5	0·5	—	0·5	0·5
Water (ml.)	0·5	—	0·5	1·0	0·25	0·75
Gas evolution (μl. CO_2/5 min.)	14	213	0	0	0	0

the apo-enzyme is obtained with an amount of codecarboxylase corresponding to 0·06 μg. C/ml. Conversion of these values into molecular concentrations must await the final purification and characterization of the coenzyme molecule. The shape of the velocity-coenzyme concentration curve would suggest that the non-protein moiety of the enzyme behaves as a coenzyme rather than as a prosthetic group.

SUMMARY

1. The lysine decarboxylase activity of coliform organisms grown in 2 % glucose-casein-digest-broth withstands drying of the organisms in acetone.

2. The enzyme can be extracted from the acetone-dried organisms by incubation with borate buffer at pH 8·5.

3. The partial purification of the enzyme is described; when *Bact. cadaveris* is used as a source, the preparation is specific for *l*(+)-lysine.

4. The enzyme is optimally active at pH 6·0, has a Michaelis constant of approximately 0·0015 M and will attack only *l*(+)-lysine, and possibly hydroxylysine, among the common amino-acids. The integrity of both amino groups in the lysine molecule is essential for activity.

5. The decarboxylation is quantitative and the product is cadaverine.

6. The enzyme can be resolved into specific apo-enzyme and a hitherto undescribed codecarboxylase by repeated precipitation with ammoniacal ammonium sulphate (66 % saturation).

7. The apo-enzyme can be reactivated by co-decarboxylase preparations but by no other co-enzyme or prosthetic group.

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Studies on Bacterial Amino-acid Decarboxylases

2. *l*(−)-TYROSINE DECARBOXYLASE FROM *STREPTOCOCCUS FAECALIS*

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This paper deals with the isolation, partial purification and some of the properties of *l*(−)-tyrosine decarboxylase from *Streptococcus faecalis*. Gale (1940*b*) showed that *l*(−)-tyrosine is the only one of 16 naturally occurring amino-acids to be decarboxylated by this organism, although it was found later that *l*-3:4-dihydroxyphenylalanine is also decarboxylated (Gale, personal communication). In the present paper an attempt is made to discover whether *l*-3:4-dihydroxyphenylalanine acts as substrate for *l*(−)-tyrosine decarboxylase or whether a separate enzyme is involved.

METHODS

Organisms. Of the faecal streptococci used in this study, one strain was isolated from human stools by Dr Crowley of the Cambridge University Pathology Department; one by Dr Cruickshank of the Group Laboratory, the North-Western Hospital; one was the laboratory stock strain; the rest were isolated by the author.

Substrates. The *l*(−)-tyrosine used for the quantitative experiments was prepared by Merck and Co.; for all other experiments it was isolated from a tryptic digest of casein and thrice recrystallized: *l*(−)- and *dl*-3:4-dihydroxyphenylalanine were supplied by Roche Products Ltd. The purification of the enzyme was worked out with *l*(−)-tyrosine as substrate, but in some cases parallel experiments were made with *l*-3:4-dihydroxyphenylalanine. The enzyme is referred to as *l*(−)-tyrosine decarboxylase throughout; data supporting the theory that a single enzyme is responsible for the decarboxylation of both these amino-acids are presented later.

Method of estimating and expressing enzyme activity. The activity of the enzyme preparations was estimated as for

l(+)-lysine decarboxylase (Gale & Epps, 1944*a*) in Warburg manometers. Unless otherwise stated, the reaction temperature was 30°, the gas phase air, the buffer 2 ml. M/5 acetate pH 5·5 and the substrate (in side-bulb) 0·5 ml. M/30 *l*(−)-tyrosine suspension or 0·5 ml. M/15 *l*-3:4-dihydroxyphenylalanine solution. Precautions were taken against progressive dissociation of the enzyme (see below) by addition to the main compartment of the manometer cup of excess (0·2 ml.) of a purified preparation of co-decarboxylase.

A unit of enzyme activity is arbitrarily defined as that amount which will liberate 100 μl. CO_2/5 min. from the appropriate substrate under the conditions outlined above. The activity is given as $Q^C_{CO_2} = \mu$l. CO_2 evolved/mg. C preparation/hr.; the rates were calculated from the CO_2 output measured at 5 min. intervals during the first 15 min. after tipping the substrate into the main reaction vessel, and C was estimated by the micro-method of Van Slyke & Folch (1940).

1. PRELIMINARY WORK ON THE PURIFICATION OF THE ENZYME

Growth of the organism and preparation of a cell-free extract. For this preliminary work the cultures were grown at 37° for 15 hr. in broth consisting of a tryptic digest of casein to which marmite (0·1 %) and glucose (1 %) had been added. As in the preparation of *l*(+)-lysine decarboxylase, the bacteria were centrifuged off, washed once and made into a thick suspension with distilled water. The suspension was cooled in ice and stirred into 10 vol. ice-cold acetone. After half an hour the precipitate was filtered off on a small Büchner funnel and washed successively with acetone, a mixture of acetone and ether in equal parts and, finally, ether. When prepared under these conditions the enzyme content of the acetone powder is apparently greater than that of the original washed suspension (Table 1); this apparent increase may be compared with that observed by Davies (1943) during extraction of acetoacetic acid decarboxylase from acetone powders of *Cl. acetobutylicum*.

l(−)-Tyrosine decarboxylase can be extracted from the acetone powder by incubation with buffer at 37°, the optimum pH being 5·5. In the preparation of *l*(+)-lysine decarboxylase from coliform organisms, extraction was favoured by a growth time of 22–24 hr., but for *l*(−)-tyrosine decarboxylase equally good results were obtained with growth periods of 12–21 hr. The quantitative nature of the process varies widely with the particular organism used in the preparation of the acetone powder. From eight strains of faecal streptococci tested the amount of enzyme going into solution varied between 20–95 % of the total; this appears to be due to strain differences and for further work an organism was chosen (*Strep. faecalis* Dunn) which gives consistent extraction of the order of 95 %. Incubation of the powder at 37° for 18–21 hr. results in optimal extraction of the enzyme without appreciable loss in activity, while incubation at room temperature for the same period is only half as effective. The rate of extraction of the enzyme is independent of the concentration of acetone powder between 10 and 50 mg./ml., but more concentrated suspensions do not centrifuge cleanly. The final conditions adopted were as follows: the powder (20 mg./ml.) was suspended in M/45 phosphate pH 5·5 and incubated at 37° for 21 hr., after which the suspension was centrifuged and the supernatant collected for further purification.

Adsorption of the enzyme. Acid-ethanol precipitation was found to be a useful step in the purification of *l*(+)-lysine decarboxylase. In the present case precipitation occurs if the enzyme is adjusted to pH 5·0 or less in the presence of 50 % ethanol, but less than 22 % of the *l*(−)-tyrosine decarboxylase activity is found in the precipitate and this treatment was abandoned. Adsorption will take place on to C_γ alumina; at pH 5·0, 72 % of the enzyme is adsorbed but the remainder is apparently destroyed; elution cannot be effected by buffers over the range pH 4–10, by $(NH_4)_2SO_4$ solutions up to 20 % saturation, or by phosphate solutions up to 2 M at pH 7·0. The adsorbed enzyme loses 75 % of its activity while standing at room temperature for an hour. Neither fuller's earth nor charcoal adsorb the enzyme under acid, neutral or alkaline conditions, and the maximum adsorption with kaolin was 20 %.

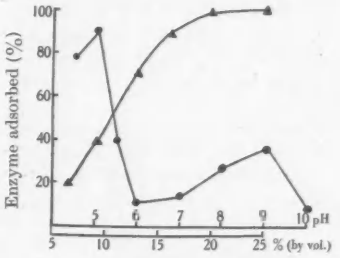

Fig. 1. Variation of adsorption on to $Ca_3(PO_4)_2$ with pH and quantity of adsorbent used. ●—● Variation with pH: 1 ml. enzyme extract adjusted to the required pH and excess $Ca_3(PO_4)_2$ suspension added: portions of precipitate taken for estimation. ▲—▲ Variation with quantity of adsorbent: 1 ml. enzyme extract + 0·2 ml. 2 M-acetate pH 5·0; different amounts $Ca_3(PO_4)_2$ suspension (30 mg./ml.) added and portions of precipitate taken for estimation. Volume of $Ca_3(Po_4)_2$ expressed as % (by vol.) of original extract.

$Ca_3(PO_4)_2$ was found to be a suitable adsorbent and Fig. 1 shows variation of adsorption with pH and with amount of $Ca_3(PO_4)_2$ added. The final conditions adopted consisted of addition to the extract of 1/5 vol. of 2 M-acetate at pH 5·0 followed

by 1/5 vol. of neutral $Ca_3(PO_4)_2$ suspension (30 mg./ml.). The enzyme is best eluted with a solution of $(NH_4)_2SO_4$; buffer solutions of range pH 4–10 and solutions of Na_2SO_4 were unsatisfactory. The optimal eluent is 1 ml. 50 % saturated $(NH_4)_2SO_4$ solution at pH 7·0/20 mg. $Ca_3(PO_4)_2$; elution was equally efficient at room temperature or at 37° over a period of 10 or 30 min.

Fractionation with ammonium sulphate. The protein is precipitated from the eluate by full saturation with $(NH_4)_2SO_4$. Owing to the small volume, this is best done by the addition of an equal volume of 50 % saturated $(NH_4)_2SO_4$ solution followed by 40 g. solid $(NH_4)_2SO_4$/100 ml. of the final solution, the resulting suspension being centrifuged at 3000 r.p.m. for 30 min. The precipitate is dissolved in distilled water and fractionated twice with saturated $(NH_4)_2SO_4$ solution, as below; further fractionation was abandoned since it led to even distribution of the enzyme among the fractions without significant increase in activity.

Repetition of adsorption on to $Ca_3(PO_4)_2$ *and elution with* $(NH_4)_2SO_4$ *solution.* After fractionation the enzyme can again be adsorbed on to $Ca_3(PO_4)_2$; the pH conditions are similar to those given above and the amount of adsorbent and eluent were related to the number of units remaining in the preparation. Optimal conditions are 75 mg. $Ca_3(PO_4)_2$/10 units enzyme for adsorption and 1 ml. 25 % saturated $(NH_4)_2SO_4$ solution/15 mg. $Ca_3(PO_4)_2$ for elution.

2. FINAL CONDITIONS FOR PURIFICATION

The strain of organism used in this preparation was *Strep. faecalis* Dunn*; Table 1 gives the volume, enzyme activity and C content at each stage of the purification.

4·5 l. of medium were inoculated, and incubated at 37° for 16 hr.; the culture was passed through a Sharples supercentrifuge and the bacteria washed and made into a thick suspension with distilled water (stage 1). The suspension was cooled in ice and stirred into 10 vol. of ice-cold acetone. After half an hour the precipitate was filtered off on a small Büchner funnel and washed successively with acetone, a mixture of acetone and ether in equal parts, and ether; it was then spread on a glass dish to dry.

The dry powder (20 mg./ml.) was suspended in M/45 phosphate pH 5·5 (stage 2 *a*) and incubated at 37° for 21 hr. At the end of this period (stage 2 *b*) the suspension was centrifuged at 3000 r.p.m. for 15 min. and the supernatant collected (stage 3). The pH of the extract was adjusted by addition of 2M-acetate pH 5·0 (1 ml. buffer to 5 ml. extract), and 3 ml. $Ca_3(PO_4)_2$ at pH 7·0 were added/10 ml. original extract; after 10 min. the precipitate was spun off and the supernatant discarded. The $Ca_3(PO_4)_2$ precipitate was washed once with distilled water before the enzyme was eluted with 50 % saturated $(NH_4)_2SO_4$ solution; the elution was carried out in two stages, the volume of eluate used each time being 1 ml./20 mg. of $Ca_3(PO_4)_2$ used for adsorption and the suspension being left to stand for 10 min. before centrifuging. The combined eluates were diluted with an equal volume of 50 % saturated $(NH_4)_2SO_4$ solution (stage 4); 40 g. solid $(NH_4)_2SO_4$ were added/100 ml. of preparation and the suspension was centrifuged at 3000 r.p.m. for 30 min.

The precipitate was dissolved in distilled water (stage 5) and sufficient saturated $(NH_4)_2SO_4$ solution added to bring to 50 % saturation (6 *a*). The precipitate was centrifuged off and the supernatant brought to 65 % (6 *b*) and then to 75 % saturation (6 *c*). The middle fraction (6 *b*) was again precipitated at 52, 58 and 63 % $(NH_4)_2SO_4$ saturation, and the precipitates were dissolved in water (stages 7 *a*, *b* and *c*).

The solution at stage 7 *b* was opalescent and faintly yellow; its pH was then adjusted by the

* Deposited in National Collection of Type Cultures: No. 6783.

Table 1. *Purification of* l(−)*-tyrosine decarboxylase* (*from* Strep. faecalis *Dunn*)

Stage	Volume (ml.)	Activity towards tyrosine (μl./ml./5 min.)	Units	Dry wt. (mg. C/ml.)	$Q^C_{CO_2}$	Activity towards dihydroxy-phenylalanine (μl./ml./5 min.)	Activity ratio: tyrosine/ dihydroxy-phenylalanine
1	28·5	1080	306	32·5	400	740	1·46
2 (*a*)	98	690	672	10·1	—	—	—
(*b*)	—	672	655	—	800	450	1·47
3	92	635	585	4·5	1,690	415	1·53
4	27·5	1570	446	3·63	5,040	1080	1·45
5	20	1720	344	3·30	6,220	1020	1·69
6 (*a*) 0–50 %	10	190	19	—	—	140	1·36
(*b*) 50–63 %	10	2600	260	1·70	18,400	1490	1·75
(*c*) 63–75 %	5	935	47	—	—	575	1·63
7 (*a*) 0–53 %	5	1590	79·5	2·42	7,900	905	1·75
(*b*) 53–58 %	5	2270	113·5	0·595	45,700	1410	1·61
(*c*) 58–63 %	5	765	37	0·67	13,700	475	1·61
8	4·5	1510	67	0·392	46,300	910	1·65

addition of 1/5 vol. 2 M-acetate pH 5·0, and $Ca_3(PO_4)_2$ added, 75 mg./10 units of enzyme. After 10 min. the precipitate was centrifuged off and washed with distilled water. The enzyme was eluted by adding 1 ml. 25 % saturated $(NH_4)_2SO_4$ solution/15 mg. of $Ca_3(PO_4)_2$ used for adsorption; after 10 min. the suspension was centrifuged and the supernatant (stage 8) collected. In this particular preparation the fractionation at stage 7 had been unusually successful and the final adsorption and elution did not result in a significant increase in enzyme activity, although the solution at stage 8 was water-clear. The final preparation (from 4·5 l. medium) contained 1·76 mg. C (*c.* 3·5 mg. protein) and the degree of purification was some 100-fold.

Ultra-violet adsorption of the enzyme preparation. A solution containing 0·267 mg. C/ml. (stage 8) in 25 % saturated $(NH_4)_2SO_4$ showed a typical protein ultra-violet adsorption curve with an extinction $\log_{10} I_0/I$ at 270 mμ = 1·6, when d = 2 cm.

3. PROPERTIES OF *l*(−)-TYROSINE DECARBOXYLASE

Source of enzyme. The enzyme preparations used in these studies were stages 8 or 7 *b* of various preparations from *Strep. faecalis* Dunn.

Variation of activity with pH *and the nature of the buffer system.* The optimum pH for the enzyme is 5·5 in acetate, phosphate or phthalate buffers, although the activity varies according to the buffer system used. pH curves for *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine in acetate and phosphate buffers are shown in Fig. 2. Discrepancies because of variation of the solubility of tyrosine with pH were avoided by keeping the tyrosine concentration that of a saturated solution at pH 5·5 (the iso-electric point) at the temperature used. The manometers were set up with 1 ml. substrate and 1·5 ml. buffer in the main compartment and 0·2 ml. enzyme, 0·1 ml. codecarboxylase preparation and 0·2 ml. buffer in the side-bulb. The activity of the enzyme at pH 5·5 was also measured in citrate and phthalate buffers (Table 2). The pH curve for the intact organism is of similar shape (Gale, 1940*b*), although the optimum varies between pH 5·0 and 5·5, whereas the optimum for enzyme preparations at all stages was 5·5. This apparent shift of the optimum pH on extraction of the enzyme from the cell was also observed by Davies (1943) for acetoacetic acid decarboxylase from *Cl. acetobutylicum* and by us for *l*(+)-lysine decarboxylase from *Esch. coli* and *Bact. cadaveris* (Gale & Epps, 1944*a*).

Table 2. *Variation of enzyme activity at* pH 5·5 *with nature of buffer system*

Buffer system	pH	μl. CO_2/5 min.
M/5 acetate	5·51	124
M/5 citrate	5·48	107
M/15 phosphate	5·48	98
M/20 phthalate	5·48	77

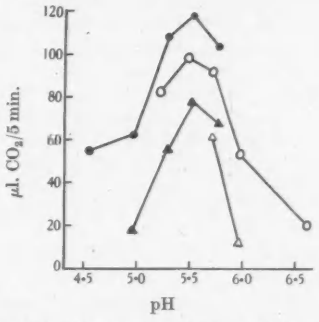

Fig. 2. Variation of enzyme activity with pH. ●—● *l*(−)-tyrosine as substrate, M/5 acetate buffer. ○—○ *l*(−)-tyrosine as substrate, M/15 phosphate buffer. ▲—▲ *l*-3:4-dihydroxyphenylalanine as substrate, M/5 acetate buffer. △—△ *l*-3:4-dihydroxyphenylalanine as substrate, M/15 phosphate buffer.

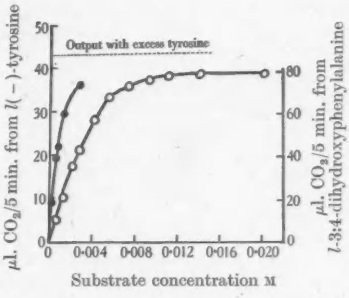

Fig. 3. Variation of enzyme activity with substrate concentration. ●—● *l*(−)-tyrosine as substrate. ○—○ *l*-3:4-dihydroxyphenylalanine as substrate (different enzyme preparation).

Effect of substrate concentration. The variation of enzyme activity with substrate concentration is shown in Fig. 3. The greatest concentration of *l*(−)-tyrosine (0·0025 M) is that of a saturated solution at pH 5·5 and 30°; in the presence of excess undissolved *l*(−)-tyrosine the rate of CO_2 production is further increased by *c.* 13 %, but this increase must depend on other factors than enzyme-substrate affinity. The Michaelis constant of such a reaction cannot be calculated from the substrate concentration curve. For *l*-3:4-dihydroxyphenylalanine the enzyme gives a normal affinity curve, the value of the Michaelis constant (the half-saturation point) being 0·0023 M.

Effect of reaction temperature. Table 3 shows the activity of the enzyme preparation at different temperatures; in order to avoid anomalies due to variation in the solubility of tyrosine with temperature the substrate consisted of 1 ml. saturated solution of *l*(−)-tyrosine at pH 5·5 and 16° (manometers set up as for Fig. 2). It appears that the activity does not increase significantly with a rise of temperature above 30°; this suggests that partial inactivation of the enzyme is taking place although the acetone powder is unaffected by prolonged incubation at 37° in the absence of substrate (Table 1) and the activity of the intact organism increases up to 44° (Gale, 1940*b*).

Table 3. *Variation of enzyme activity with temperature*

Temp. (°)	Activity towards tyrosine (μl./ml./5 min.)	Activity towards dihydroxyphenylalanine	Activity ratio: tyrosine / dihydroxyphenylalanine
16·5	25·5	16·5	1·55
25	50	28·5	1·75
31	69	40	1·73
38	70	41	1·71

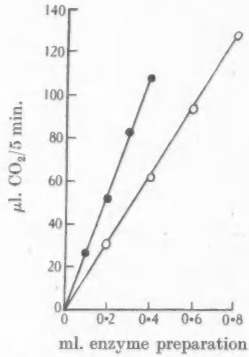

Fig. 4. Variation of activity with enzyme concentration. •—• *l*(−)-tyrosine as substrate. o—o *l*-3:4-dihydroxyphenylalanine.

Effect of enzyme concentration. The rate of CO_2 production from both substrates is directly proportional to the amount of enzyme used over the range included in manometric tests (Fig. 4).

Quantitative nature of the reaction and stereospecificity of the enzyme. Manometers were set up containing 0·5 ml. M/30 solution (or suspension) of the appropriate substrate and the course of the reaction followed. Since pH 5·5 is near the limit of effective buffering power of acetate, M/5 citrate was used in these experiments. The results are tabulated in Table 4 from which it appears that decarboxylation is quantitative and that the enzyme is specific for the laevo-isomers.

Isolation of reaction products. A Krebs's vessel incubated at 30° contained 30 ml. M/60 *l*(−)-tyrosine suspension, 30 ml. M/5 acetate pH 5·5 and 3 ml. enzyme preparation. The course of the reaction was followed in a control manometric experiment until gas evolution ceased (*c.* 90 min.); at the end of this period the contents of the Krebs's vessel were deproteinized with ferric sulphate, acidified to pH 1, concentrated *in vacuo* to a small volume and treated with benzoyl chloride as described by Gale (1940*b*). The resulting dibenzoyltyramine was twice recrystallized from ethanol. The product (120 mg.) melted at 170–171° (corrected) and gave on analysis (Weiler) C, 75·87; H, 5·54; N, 3·97%. Calc. for $C_{22}H_{19}NO_3$: C, 76·50; H, 5·54; N, 4·06%.

In another experiment, 30 ml. M/30 *l*-3:4-dihydroxyphenylalanine solution replaced the *l*(−)-tyrosine and 6 ml. enzyme preparation were used. At the end of the reaction period zinc hydroxide was used as a deproteinizing agent, since here boiling the solution with ferric sulphate led to formation of coloured oxidation products. The deproteinized solution was benzoylated with sufficient benzoyl chloride to give the tribenzoyl derivative. The tribenzoyldihydroxyphenylethylamine was thrice recrystallized from ethanol. Yield 94 mg., m.p. 140–141° (corrected). Analysis (Weiler), C, 78·20; H, 5·28; N, 3·28%. Calc. for $C_{29}H_{23}NO_5$: C, 79·50; H, 5·40; N, 3·44%.

Specificity, inhibition and denaturation. It has already been stated that *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine are the only naturally occurring amino-acids to be decarboxylated by *Strep. faecalis*; the following compounds chemically related to tyrosine were tested as substrates for the enzyme preparation and as inhibitors of its reaction

Table 4. *Quantitative nature of decarboxylation of* l(−)-*tyrosine and* l-3:4-*dihydroxyphenylalanine and stereospecificity of enzyme preparation*

Results refer to total CO_2 output in μl. after subtraction of the blank (0·5 ml. M/30 substrate = 373 μl. CO_2).

Substrate	No. of experiments	Mean	Standard deviation	Range	% of calc.
l(−)-Tyrosine	9	357	11	343–372	96
l-3:4-Dihydroxyphenylalanine	5	148	3	343–353	94·5
dl-Tyrosine	11	174	9	172–192	47
dl-3:4-Dihydroxyphenylalanine	5	171	4	167–177	46

with *l*(−)-tyrosine: *l*-phenylalanine, *l*-3:4-dihydroxyphenylalanine, *l*-diiodotyrosine, *l*-tyrosine sulphonic acid, N-methyl-*dl*-tyrosine, methoxy-*l*-tyrosine, N-methyl-methoxy-*l*-tyrosine, *l*-thyroxine, *l*-thyronine (desiodothyroxine) and glycyl-tyrosine. *l*-3:4-Dihydroxyphenylalanine was attacked by the preparation; none of the others had any action either as substrate or inhibitor.

Table 5 shows the effect of general inhibitors; in those cases where an effect was observed, dilution below the molar concentrations whose negative logarithms are given in the table resulted in a lesser degree of inhibition. $(NH_4)_2SO_4$ in low concentrations has been found to have an inhibitory effect on the activity of preparations of *l*(+)-lysine decarboxylase, but solutions of $(NH_4)_2SO_4$ up to 33% saturation in strength do not significantly affect the activity of *l*(−)-tyrosine decarboxylase.

Table 5. *Effect of enzyme inhibitors*

Inhibitor	Substrate: tyrosine		Substrate: dihydroxyphenylalanine	
	$-\log_{10}$ (molar conc.)	% inhibition	$-\log_{10}$ (molar conc.)	% inhibition
Ag (as $AgNO_3$)	2	99	2	100
Cu (as $CuSO_4.5H_2O$)	3	98	3	97
Hg (as $HgCl_2$)	2	97	2	96
Fe (as $Fe(NH_4)_2(SO_4)_4.6H_2O$)	1*	99	1*	98
Fe (as $FeCl_3$)	2	99	2	100
Pb (as $Pb(CH_3COO)_2.3H_2O$)	1	98	1	98
NaF	1	0	1	0
Iodoacetate	1	48	1	45
Na_2S	2	0	2	0
KCN	2	93	2	95
NaN_3	1	13	1	15
NH_2OH	4	97	4	97
N_2H_4	3*	98	3*	98
Baeyer 205 (antrypol)	2	75	2	73
Sulphanilamide	2	9	2	10
p-Phenylenediamine	2	41	2	40

* These experiments were carried out in an atmosphere of N_2, since dihydroxyphenylalanine took up O_2 even in absence of enzyme when these inhibitors were present.

Table 6 shows the effect of various treatments on the activity of the enzyme; the losses in activity are irreversible and accompanied by protein denaturation. Where necessary, the pH of the preparation was readjusted to 5·5 before samples were taken for that deterioration begins. The isolation from brewer's yeast of a compound with the function of codecarboxylase for *l*(+)-lysine decarboxylase is described in a further paper in this series (Gale & Epps, 1944*b*); it was found that addition of this compound restored the activity of the *l*(−)-tyrosine decarboxylase preparations, whose deterioration was probably caused by the spontaneous and progressive dissociation of the enzyme into apo-enzyme and codecarboxylase.

Table 6. *Denaturation of the enzyme*

Conditions of treatment	Activity remaining (%)	
	Substrate: tyrosine	Substrate: dihydroxyphenylalanine
55° for 5 min. (pH 5·0)	57	53
60° for 5 min. (pH 5·0)	19	21
pH 4·5 for 10 min.	64	63
50% ethanol at pH 5·0 (10 min.)	6	6
10^{-3} M-$KMnO_4$ at pH 5·5 (10 min.)	76·5	73
$NaNO_2$ (0·1 M) at pH 5·5 (10 min.)	44	46

At stage 8 of the preparation (above) the enzyme complex was 50% dissociated, but more complete dissociation could be induced by suitable treatment; this consisted either in precipitating the protein at stage 6 *b* with 3 vol. of saturated $(NH_4)_2SO_4$ solution containing 3·3% ammonia (cf. *l*(+)-lysine decarboxylase, Gale & Epps, 1944*a*) or in keeping the enzyme preparation in the ice-chest for 72 hr. and then dialyzing it overnight against glass-distilled water; in the former case about half the enzyme activity is lost by irreversible inactivation, while in the latter recovery is nearly quantitative on addition of the coenzyme.

Addition of boiled yeast or codecarboxylase preparation restores the activity of the apo-enzyme

Dissociation of the enzyme. During the early stages of this work it was observed that the activity of the enzyme preparations decreased rapidly during purification, so that the quantitative recovery from any stage was poor. This phenomenon does not occur with the intact organism nor with the dry acetone powder, which maintains its full activity for several months; it is only after extraction of the enzyme

Table 7. *Restoration of activity to apo-enzyme*

Manometer no.	1	2	3	4	5	6	7	8	9
	Additions in ml.								
Apo-enzyme	0·2	0·2	0·2	0·2	—	—	—	0·2	0·2
Codecarboxylase preparation	—	0·2	—	0·2	0·2	0·2	—	—	0·2
M/30 Tyrosine	—	—	0·5	0·5	0·5	—	0·5	—	—
M/15 Dihydroxyphenylalanine	—	—	—	—	—	—	—	0·5	0·5
M/5 Acetate pH 5·5	2·8	2·6	2·3	2·1	2·3	2·8	2·5	2·3	2·1
CO_2 output, μl./5 min.	0	0	17	126	0	0	0	4	82

towards both *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine (Table 7), but none of the following had any significant effect: coenzyme I, coenzyme II preparation, cocarboxylase, aneurin, flavin adenine dinucleotide, flavin mononucleotide, riboflavin, muscle adenylic acid, adenosine, nicotinamide, nicotinic acid. Fig. 5 shows the activity obtained

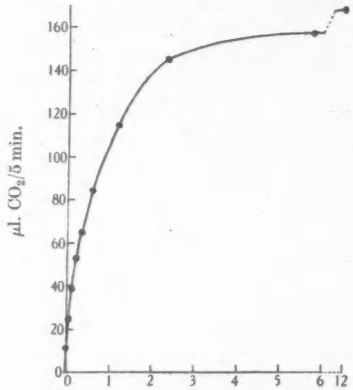

Fig. 5. Restoration of activity to apo-enzyme *l*(−)-tyrosine as substrate.

when varying amounts of a purified preparation of codecarboxylase were added to the *l*(−)-tyrosine decarboxylase apo-enzyme; the shape of the curve suggests a loose association of the coenzyme type rather than a prosthetic group linkage and half-saturation of the enzyme is given by 0·60 μg. C of codecarboxylase/ml. The distribution of the co-decarboxylase of *l*(+)-lysine decarboxylase and its probable identity with the coenzyme of *l*(−)-tyrosine decarboxylase are dealt with elsewhere (Gale & Epps, 1944*b*).

4. *l*(−)-TYROSINE AND *l*-3:4-DIHYDROXYPHENYLALANINE AS ALTERNATIVE SUBSTRATES FOR *l*(−)-TYROSINE DECARBOXYLASE

Holtz, Credner & Walter (1940) and Blaschko (1942) have shown that in animal tissues *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine are decarboxylated by different enzymes. Does the same hold for bacteria or is a single enzyme concerned? The evidence can be summarized as follows: 1. The activity towards both substrates of the preparation described above was estimated during purification to an 100-fold extent, and there was no significant difference between the values obtained for the activity ratios (for the values in the last column of Table 1, mean = 1·58, standard deviation = 0·11). 2. The response of the activities of enzyme preparations towards *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine with changes in pH, temperature and enzyme concentration are shown in Fig. 2, Table 3 and Fig. 4 respectively; there is general similarity between the pairs of results. The variation of activity with substrate concentration is shown in Fig. 3. 3. The effects of general inhibitors are very similar towards the two substrates (Table 5). 4. The effects of exposing the enzyme to various denaturing processes are similar towards the two activities (Table 6). 5. When the concentration of each substrate was sufficient to saturate the enzyme, the rate of CO_2 production from a mixture of *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine was intermediate between those recorded for each substrate alone (Table 8). 6. Gale (1940*a*, *b*; 1941) showed that *l*(−)-tyrosine can be decarboxylated only by *Strep. faecalis* and *Cl. aerofoetidum* among numerous organisms tested. From a survey of several of the same organisms it appears that only *Strep. faecalis* and *Cl. aerofoetidum* possess activity towards *l*-3:4-dihydroxyphenylalanine. An acetone powder was prepared from a culture of *Cl. aerofoetidum* grown anaerobically overnight at 37° in 2% glucose broth to which meat had been added (Gale, 1941). The pH curves were determined for the decarboxylation of *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine (Fig. 6). At the optimum pH (5·5) the activity ratio was 2·7. The variation of enzyme activity with substrate concentration was determined (Fig. 7); the Michaelis constant for *l*-3:4-dihydroxyphenylalanine is 0·0012 M. The result of a mixed substrate experiment is shown in Table 8.

These observations show that there is close similarity between the decarboxylation of *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine by bacteria, and that the criteria laid down by Dixon (1938) for the identity of two enzymes are satisfied. Further evidence for a single enzyme being concerned is provided by the restricted and identical distribution of

Table 8. *Mixed substrate experiments*

(Substrates, 0·5 ml. M/30 *l*-tyrosine suspension and 0·5 ml. M/15 *l*-3:4-dihydroxyphenylalanine solution; activity as μl. CO_2/5 min.)

	Tyrosine	Dihydroxyphenylalanine	Tyrosine + dihydroxyphenylalanine
0·2 ml. Enzyme preparation (*Strep. faecalis* Dunn)	106	58	93
20 mg. Acetone powder (*Cl. aerofoetidum*)	57	21	43

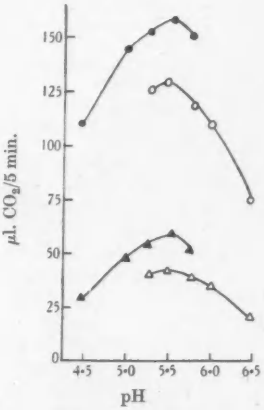

Fig. 6. Variation of activity with pH: *l*(−)-tyrosine decarboxylase from *Cl. aerofoetidum* as for Fig. 4, with 50 mg. acetone powder in each manometer vessel. ●—● *l*(−)-tyrosine as substrate, M/5 acetate buffer. ○—○ *l*(−)-tyrosine as substrate, M/15 phosphate buffer. ▲—▲ *l*-3:4-dihydroxyphenylalanine as substrate, M/5 acetate buffer. △—△ *l*-3:4-dihydroxyphenylalanine as substrate, M/15 phosphate buffer.

the two activities throughout certain bacterial species, by the constancy of the activity ratio during purification of the enzyme preparation, by the similarity of the effects of changes in experimental temperature, by the similar action of a wide range of inhibitors and denaturing agents and by the results of mixed substrate experiments. The difference in the activity ratios and Michaelis constants of enzyme preparations from *Strep. faecalis* and *Cl. aerofoetidum* is not necessarily surprising when the different nature of these organisms is considered. Taking all these factors together, it seems highly probable that only one enzyme is concerned in the

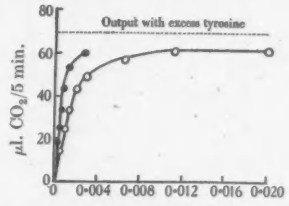

Fig. 7. Variation of activity with substrate concentration: *l*(−)-tyrosine decarboxylase from *Cl. aerofoetidum*. Each manometer vessel contained, for *l*(−)-tyrosine, 2·3 ml. M/5 acetate pH 5·5 and 0·2 ml. acetone powder suspension (100 mg./ml.). 2·1 ml. buffer and 0·4 ml. enzyme were used with *l*-3:4-dihydroxyphenylalanine. 0·5 ml. of the appropriate substrate was contained in the side-bulb. ●—● *l*(−)-tyrosine as substrate. ○—○ *l*-3:4-dihydroxyphenylalanine.

decarboxylation of *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine by bacteria.

SUMMARY

1. The extraction, purification and properties of *l*(−)-tyrosine decarboxylase from *Strep. faecalis* are described.

2. The enzyme dissociates spontaneously during purification, and preliminary evidence is presented that the coenzyme involved is the same as that of *l*(+)-lysine decarboxylase.

3. The enzyme preparations are specific for *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine, which they decarboxylate quantitatively to the corresponding amines.

4. The evidence suggests that, in contrast to the mammalian decarboxylases, a single enzyme is responsible for the bacterial decarboxylation of *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine.

I wish to express my thanks to Dr E. F. Gale and Dr M. Stephenson for their constant encouragement and help during this work. I am grateful to Prof. A. C. Chibnall and to Dr M. Dixon for valuable criticism and to the Medical Research Council for a whole-time grant.

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Studies on Bacterial Amino-acid Decarboxylases

3. DISTRIBUTION AND PREPARATION OF CODECARBOXYLASE

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Studies of the *l*(+)-lysine and *l*(−)-tyrosine decarboxylases have shown that these enzymes can be reversibly resolved into a specific protein portion and a hitherto undescribed coenzyme (Gale & Epps, 1944; Epps, 1944). The present communication deals with the distribution and isolation of this coenzyme substance which we have tentatively called 'codecarboxylase'.

METHODS

Figures have been published showing the variation of velocity of decarboxylation with the concentration of codecarboxylase for a given amount of apo-enzyme in the cases of *l*(+)-lysine decarboxylase (Gale & Epps, 1944) and of *l*(−)-tyrosine decarboxylase (Epps, 1944). In either case the velocity of decarboxylation bears an approximately linear relation to the amount of codecarboxylase added as long as this amount is considerably less than the saturation value for the apo-enzyme used. Consequently this velocity can be used as a measure of the amount of coenzyme present during test, and comparative measurements can be made as long as other conditions remain constant and the same amount of the same preparation of apo-enzyme be used for any one series of comparisons.

We have used this as a basis for the investigation of distribution and purification of codecarboxylase. The rate of decarboxylation is measured manometrically as previously described. In these studies we have made measurements with the apo-enzymes of both lysine decarboxylase and tyrosine decarboxylase, so that a double check should be provided on the results and also on evidence as to the identity of the coenzyme for these two enzymes. Sufficient enzyme was prepared in both cases to cover each full series of results; the apo-enzyme portions were prepared and made up in solutions of such strength that 1 ml. should contain 8 units of enzyme when saturated with coenzyme. 0·5 ml. of this preparation was used per test and measured portions of the coenzyme preparations were taken, such that the CO_2 evolution/5 min. was less than 200μl., thus ensuring that the rate fell on the linear part of the velocity-coenzyme concentration curve. Tests were carried out at 30° and at the optimum pH of 6·0 for lysine, or 5·5 for tyrosine, decarboxylase. An arbitrary unit of coenzyme is defined as that amount of coenzyme which increases the rate of decarboxylation by the apo-enzyme by 100μl./5 min. As a measure of the amount of substance in preparations, the C content was estimated by the wet combustion method of Van Slyke & Folch (1940). The activity, P, of coenzyme preparations is defined as μl. CO_2 liberated from substrate, due to presence of coenzyme/hr./mg. C of coenzyme. P is calculated from the formula $P=12\,(R-r)/W$, where $r=\mu$l. CO_2 liberated/5 min. in presence of apo-enzyme alone, and $R=\mu$l. CO_2 liberated/5 min. in presence of apo-enzyme plus W mg. C of coenzyme preparation, provided that the value of R falls within the limits defined above. The values of P, and number of units, can be used only for comparative purposes, for tests carried out with a standard amount of a given apo-enzyme preparation.

DISTRIBUTION OF CODECARBOXYLASE

A survey of various animal, plant, yeast and bacterial cells has been made on the assumption that the active principle in each case is the same substance ('codecarboxylase'). A suitable amount of the tissue to be assayed was ground as finely as possible in water, and the suspension was then boiled for 2 min. and again ground. Measured portions were taken for estimation of the coenzyme activity, by addition to apo-enzyme, and of C content. In the manometric estimation of coenzyme activity it is necessary to equilibrate for a constant time, not less than 15 min., before the addition of substrate. Table 1 gives results obtained with both lysine and tyrosine apo-decarboxylases, expressed as units of coenzyme/mg. C of tissue, placed in descending order of activity.

The table shows the wide distribution of coenzyme activity. Positive results have been obtained with all the rat tissues tested; with all bacteria tested, whether possessing potential amino-acid decarboxylases (as *Esch. coli*, *Strep. faecalis*, etc.; Gale, 1940) or not (as *Ps. aeruginosa*; Gale, 1942), with *Sarcina lutea*, etc.; brewer's and baker's yeasts; and with the two plant tissues tested. The only biological substance found to give completely negative results was urine. In some cases (those marked with an asterisk in the table) the assays are only very approximate, as the impossibility of making homogeneous suspensions makes it very difficult to measure out accurate portions for assay. The substances are listed in the order of their activity towards lysine decarboxylase but it can be seen that, with few exceptions, the order of activities is the same when the coenzyme activity is assayed with regard to tyrosine decarboxylase. Amongst the substances having an activity towards lysine decarboxylase of more than 1 unit/mg. C, the most easily obtainable under present circumstances is

brewer's yeast and this has been selected as the most convenient source for isolation of the co-decarboxylase.

Table 1. *Distribution of codecarboxylase in various tissues, cells and fluids*

Tissue	Carbon (mg./ml.)	Lysine codecarboxylase Units/ml.	Lysine codecarboxylase Units/mg. C	Tyrosine codecarboxylase Units/ml.	Tyrosine codecarboxylase Units/mg. C
Rat skeletal muscle	3·65	5·6	1·54*	1·19	0·325*
B. subtilis	2·85	4·3	1·5	1·7	0·6
Staph. aureus	1·72	2·55	1·48	0·6	0·35
Rat liver	4·2	5·1	1·22	1·52	0·36
Esch. coli	5·65	6·3	1·12	2·1	0·37
Brewer's yeast	11·6	11·9	1·03	3·4	0·29
Ps. aeruginosa	4·45	3·4	0·765	1·3	0·29
Rat kidney	6·3	4·0	0·63	1·0	0·16
Sarcina lutea	4·2	1·7	0·41	0·65	0·155
Rat heart	4·8	1·85	0·385*	0·51	0·106*
Rat lung	2·1	0·80	0·38	0·22	0·105
Baker's yeast	9·3	3·15	0·34	1·1	0·12
Brassica oleracea	1·16	0·40	0·29*	0·1	0·07*
Pisum sativum	5·25	1·25	0·24*	0·34	0·065*
Rat gut	7·3	1·65	0·23*	0·45	0·062*
Rat brain	13·7	2·9	0·21	0·9	0·065
Strep. faecalis	5·4	0·7	0·13	0·18	0·033
Milk (cow)	49·8	2·15	0·043	0·61	0·012
Blood (rat)	24·3	0·9	0·037*	0·11	0·0045*
Urine (man)	—	—	—	—	—

* These analyses are approximate only (see text).

PRELIMINARY WORK ON PREPARATION OF CODECARBOXYLASE FROM DRIED BREWER'S YEAST

1. *Extraction from yeast.* An aqueous suspension of brewer's yeast, boiled for 5 min., is a good source of coenzyme; but if the suspension is centrifuged most of the activity is carried down with the sediment. If the yeast is boiled in N/10 HCl no activity is extracted from the sediment; while if the boiling is in N/10 NaOH, there is some loss, but about half the activity remains in the supernatant liquid after removal of the sediment. The extraction of coenzyme from the cell is thus assisted by mildly alkaline conditions. Table 2, A, shows the effect of NaOH concentration upon the extent of extraction at 100° for 15 min.; concentrations above N/10 NaOH appear to have a destructive effect. By the use of material extracted in this way, it was found that the Ba salt of the coenzyme is soluble in water, while much inactive material can be precipitated by the addition of barium salts. We thought therefore that a step in the purification could be combined with the extraction process, if the latter were made with dilute barium hydroxide solution instead of NaOH. Table 2, B, shows the extraction obtained by boiling 1 g. dried yeast in 5 and 10 ml. $Ba(OH)_2$ solutions of various strengths, and the results show that 10 vol. N/28 $Ba(OH)_2$ extract twice as much as 5 vol., while stronger solutions appear to be destructive. Study of the time of extraction in N/28 $Ba(OH)_2$ (Table 2, C) shows that rapid destruction of coenzyme activity takes place if the heating process is prolonged for more than a few minutes. Consequently we tested the effect of cold extraction of brewer's yeast which had been dried at room temperature and then ground to a fine powder. Table 2, D, shows that extraction at room temperature in N/18 $Ba(OH)_2$ is the most efficient process so far employed and that there is no loss on standing for 30 min. Later experience has shown that satisfactory extraction is consistently obtained by the use of 10 vol. 0·75% $Ba(OH)_2.8H_2O$, on standing for 30 min. at room temperature, and that there is no significant deterioration in activity if the mixture is left for 3–4 hr. at room temperature.

Table 2. *Conditions for extraction of codecarboxylase from dried brewer's yeast*

A. *Extraction with* NaOH *at* 100°

(Tubes contained 1 g. dried yeast in 5 ml.; incubated at 100° for 15 min. and sediment centrifuged down; 0·2 ml. supernatant for test.)

Conc. NaOH ...	N/5	N/10	N/15	N/20	N/50
Units of coenzyme/ml. extract	2·95	4·80	4·40	3·45	2·40

B. *Extraction with* $Ba(OH)_2$ *at* 100°

(Tubes contained 1 g. dried yeast in (*a*) 5 ml., (*b*) 10 ml.; incubated at 100° for 15 min. and sediment centrifuged down; 0·2 ml. supernatant taken for test.)

Conc. $Ba(OH)_2$ ...		N/3·7	N/9	N/18	N/28	N/37
Units of coenzyme/ml. extract:	(*a*)	—	3·4	3·0	2·4	2·5
	(*b*)	0·4	1·5	1·6	2·55	—

C. *Time of extraction with* N/28 $Ba(OH)_2$ *at* 100°

(Extraction with 10 vol. N/28 $Ba(OH)_2$, with heating at 100° before centrifuging.)

Time of heating (min.)	1	5	10	15	20	25	30	45	60
Units of coenzyme/ml. extract	5·4	4·4	3·2	2·5	1·6	1·1	0·98	0·55	0·4

D. *Extraction with* $Ba(OH)_2$ *at room temperature*

(In each case 5 g. dried yeast were ground up with 50 ml. $Ba(OH)_2$ solution at room temperature; left for (*a*) 5 min., (*b*) 30 min. and sediment then centrifuged down; 0·2 ml. supernatant taken for test.)

Conc. $Ba(OH)_2$ ...		N/9	N/18	N/28	N/55	0
Units of coenzyme/ml. extract:	(*a*)	—	—	3·9	—	—
	(*b*)	4·20	7·80	4·36	0·94	0·64

2. *Barium salt.* The Ba salt of the coenzyme is soluble in water but is precipitated by alcohol (ethanol or methanol). Addition of alcohol to the crude $Ba(OH)_2$ extract results in the precipitation of the coenzyme accompanied by much inactive

material, including a certain amount of protein-like substance. It is better to remove this protein-like material by alcohol precipitation first. Consequently the crude $Ba(OH)_2$ extract is first treated with H_2SO_4 to remove the Ba as sulphate and the material is then poured into alcohol to remove the protein. The latter will flocculate cleanly only if the pH lies between 6·8 and 7·0; hence the extract is first acidified to pH 3 with 25% H_2SO_4, left to stand for about 15 min. and the pH then readjusted to 6·8 with 10% NaOH. On pouring the mixture into alcohol, the $BaSO_4$ is carried down with the protein, leaving the active substance in the alcoholic supernatant liquid. The active substance can now be precipitated from the alcohol by the addition of concentrated barium acetate solution. Washing of the precipitate so obtained does not remove the active material, which appears to be firmly adsorbed on the precipitate, and some purification can be achieved at this stage by exhaustive washing of the precipitate with hot water. After such treatment the active substance can be redissolved by decomposing the precipitate with N/10 H_2SO_4. Table 3

Table 3. *Optimum concentration of alcohol for precipitation of* Ba *salts*

(Dried yeast extracted with 10 vol. 0·75% $Ba(OH)_2.8H_2O$ at room temperature; sediment centrifuged down; supernatant adjusted to pH 3 with 25% H_2SO_4; left to stand 15 min. and readjusted to pH 6·8 with 10% NaOH. 2 ml. portions taken, poured into alcohol and precipitate centrifuged down and discarded; saturated Ba acetate added to supernatant until precipitation complete; precipitate washed in 10 ml. water; precipitate decomposed in 1 ml. N/10 H_2SO_4; vol. adjusted to 2 ml., and 0·1 ml. taken for test.)

Activity of initial crude extract = 12·3 units codecarboxylase/ml.

Vol. methanol used for precipitation	Units co-enzyme/ml.	Vol. ethanol used for precipitation	Units co-enzyme/ml.
1	10·9	1	10·6
2	10·9	2	9·8
3	9·4	3	8·4
4	5·3	4	3·5
5	4·1	5	2·1

shows the effect of ethanol concentration upon the yield of coenzyme, from the crude extract stage to the decomposition of the washed precipitate. Methanol gives better yields and cleaner precipitates than ethanol and the highest yields are obtained by the use of twice the volume of methanol. A volume of either ethanol or methanol equal to the volume of aqueous solution gives equally good yields, but removes little protein in the first precipitation, so that the Ba precipitate contains much inactive material.

3. *Lead and silver salts.* Both the Ag and Pb salts of the coenzyme are insoluble at pH 7 in water, but decomposition of these salts with H_2S leads to adsorption of the coenzyme on the metallic sulphides. No efficient method of elution has been discovered. The active material can be regenerated from the Pb salt by decomposition with H_2SO_4 and from the Ag salt with HCl, but the high sensitivity of the enzymes to the presence of Ag (Gale & Epps, 1944; Epps, 1944) makes this latter procedure unsatisfactory. Table 4 shows that precipitation of the Pb salt begins about pH 5·5 but is not complete until neutrality is reached; the Ag salt is precipitated at neutrality also.

Table 4. *Precipitation of lead and silver salts of codecarboxylase*

(Extract adjusted to pH 1 with HNO_3, and excess Pb acetate added to 2 ml. portions; pH then raised in separate samples and precipitates collected at pH 3, 5·5, 6·5, 7·0 and 8·0 respectively. Precipitates decomposed in 1 ml. N/10 H_2SO_4; 1 ml. water added and 0·2 ml. taken for test.)

Precipitation at pH ...	3	5·5	6·5	7·0	8·0
Units of coenzyme/ml. after decomposition of Pb salt	0	0·12	0·48	0·48	0·52

(Experiment repeated, using $AgNO_3$ as precipitating agent. In this case the precipitates were centrifuged off and the supernatant treated with NaCl to remove Ag, neutralized, and the volume adjusted; 0·25 ml. of supernatant taken for test.)

Precipitation at pH ...	3	4	6	7·5	9·0
Units of coenzyme/ml. of supernatant	0·5	0·5	0·45	0	0

4. *Alumina C_γ adsorption.* From crude extracts adsorption of the active material occurs on alumina C_γ suspension at pH 4·0. Elution has not been achieved. More highly purified preparations can be eluted by strong phosphate at pH 7·0, but there is no significant purification.

5. *Solubility in organic solvents, etc.* The active material can be extracted from acid solution with *n*-butanol, *iso*butanol, phenol, *m*- or *p*-cresol but not with amyl alcohol. The partition coefficient between phenol and water is increased in favour of the phenol by saturation of the water phase with ammonium sulphate. The active material is precipitated by phosphotungstic acid but not by picric or picrolonic acid.

METHOD OF PURIFICATION OF CODECARBOXYLASE FROM DRIED BREWER'S YEAST

Table 5 shows the steps in the preparation of codecarboxylase adopted as a result of the preliminary investigations described. The yields and degree of purification over each stage were followed by measuring units of codecarboxylase and *P* values under standard conditions, with both lysine apo-

decarboxylase and tyrosine apo-decarboxylase. As each step was completed, measurements were made of the volume of the preparation, its activity as codecarboxylase with both apo-enzymes, and its C content. The steps in the preparation of codecarboxylase from 1 kg. of dried brewer's yeast are as follows.

1. 10 l. of 0·75 % $Ba(OH)_2.8H_2O$ solution are prepared by gentle warming. The dried and powdered yeast (1 kg.) is then added in small amounts with vigorous stirring to form a homogeneous suspension. The operation is spread over about 30 min., any lumps being broken up with the fingers. The addition of a few drops of caprylic alcohol as wetting agent is helpful.

2. The suspension is then centrifuged. We have used an International 13-l. serum centrifuge for this purpose, spinning for 15 min. at 2000 r.p.m. The sediment occupies about one-fifth of the total volume after centrifuging, and the collected supernatant is a slightly turbid, brown liquid. 25 % H_2SO_4 is added to the supernatant until the pH is approx. 3 (bright yellow to bromocresol-green). The mixture is allowed to stand 15–30 min. at this stage and is then neutralized with 10 % NaOH to approx. pH 6·8 (light green to bromothymol-blue). There is no need to remove the precipitate formed by this procedure. The mixture is now poured into 2 vol. methanol and stirred vigorously. A thick precipitate forms which flocks and settles within a few minutes. If the supernatant is cloudy, stirring the sediment and allowing it to settle again usually results in a perfectly clear yellow-brown supernatant which can be decanted. The settled sediment can be filtered under a small negative pressure on large Büchner funnels. Saturated barium acetate solution (usually 250–300 ml.) is now added with stirring to the clear alcoholic filtrate until precipitation is maximal. A fine precipitate forms which settles slowly. After 2–3 hr. the supernatant liquid can be decanted and discarded, and the sludge separated on a Sharples super-centrifuge. The material is stable from this stage onwards. The precipitated material is broken up in *c.* 1 l. hot distilled water to form a fine homogeneous suspension and centrifuged off again. The supernatant liquid is coloured yellow and the washing process is continued until the washings are colourless; five washings, with *c.* 600 ml. boiling water each time, are usually required. The washed precipitate is then suspended in *c.* 300 ml. N/2 H_2SO_4 and left to decompose for about 30 min. On centrifuging, a reddish supernatant is obtained which contains the active material. The white sediment is resuspended in 200 ml. N/2 H_2SO_4, centrifuged as before and the two supernatants collected. Table 5 shows that the whole of this step results in a purification of about 30 times with little loss of active material.

3. Phenol is melted with approx. 10 % water. The acid extract from (2) is saturated with this phenol and then extracted 4 times with 20 ml. liquid phenol. This extraction seems to remove most of the flavin nucleotides, as the first extracts are coloured deep red and, if carried through the later steps, give fluorescent yellow solutions having the properties of flavin compounds. Some coenzyme is extracted at this stage, but reaches a limit at 7000–8000 units (lysine) beyond which no further extraction takes place. It has been found most satisfactory to discard this first extract, as little purification is obtained by its inclusion in the next stage. Some of the lost coenzyme can be recovered by shaking the phenol extract with one-tenth vol. water and adding the water extract to the aqueous mother-liquor.

4. The mother-liquor from the first phenol extraction is now saturated with $(NH_4)_2SO_4$ and re-extracted with liquid phenol: the saturation should be carried out in the presence of phenol and it is advisable to use good quality $(NH_4)_2SO_4$. Four extractions, each with *c.* 50 ml. phenol, will usually extract about 50 % of the remaining active material, but further extraction is difficult. It has been found inadvisable to increase the scale of the preparation at this stage, as the percentage extraction decreases with the volume of the mother-liquor. The active material is removed from the phenol by the addition of 3 vol. ether and vigorous shaking with three or four successive amounts of 30 ml. water. The water layer becomes coloured yellow and extraction should be continued until the phenol-ether layer is colourless. The yields over this step seldom exceed 50 % but a purification of 3–4 times is effected. The water extract is washed twice with equal volumes of ether to remove residual phenol.

5. The water extract is acidified with HNO_3 to pH 1 (red to *m*-cresol-purple) and extracted 10–12 times with an equal volume of *iso*butanol saturated with water. The butanol extract is coloured light yellow, while most of the coloured material remains in the water phase. Extraction with *n*-butanol results in more rapid and quantitative extraction but little purification, while *iso*butanol is far more specific in its extraction. The *iso*butanol extracts are combined, neutralized with NaOH to pH 7·0 (green to bromothymol-blue) and then extracted with 50 ml. N/10 NaOH. The coloured material and coenzyme activity pass quantitatively into the alkali, which separates cleanly only if the preliminary adjustment to pH 7·0 has been accurate—the emulsion which otherwise results is best separated by centrifuging. The yields from the *iso*butanol extraction vary from 50 to 65 % with a purification of 2–3 times.

6. The alkaline extract is adjusted to pH 7·0 with HNO_3 and excess lead acetate is added, when the coenzyme is precipitated as its lead salt: it may

be necessary to readjust the pH to 7·0 with dilute alkali after the addition of the lead acetate. Precipitation is complete in 10–15 min. Some inactive yellow material is not carried down under these circumstances and decomposition of the lead precipitate shows purification with little loss.

7. The lead salt is decomposed in N/10 H_2SO_4 and the excess sulphate removed with barium acetate. Whenever $BaSO_4$ is precipitated in the presence of coenzyme, some active material is lost by adsorption. This is greatest if precipitation takes place at pH 5–6 and least in conditions more strongly acid than pH 1. There is little loss at this stage of the preparation but some purification results from absorption of pigment. The $BaSO_4$ is centrifuged off and washed, the washings being added to the first centrifugate.

8. Excess of barium acetate is added, the pH is adjusted to 7·0 and the barium salts are fractionated with ethanol. The concentration is made up to 30 % (by vol.) ethanol and the solution is left 30–60 min. to complete precipitation. The precipitate (8 *a*) is spun off and alcohol added to 50 % ethanol. After 60 min. the resulting precipitate (8 *b*) is spun off and the process repeated at 70 % ethanol (precipitate 8 *c*). In each case the precipitate is redissolved in water, adjusted to pH 6·8 and reprecipitated as the lead salts. The lead precipitates are washed and decomposed in N/10 H_2SO_4 for the activity test—this avoids the presence of 'foreign' C in the preparations for C analysis. Table 5 shows that the most active fraction is that precipitated by 30 % ethanol and that the yield represents some 40–50 % of the active material present at stage 7.

9. A further fraction, slightly less active than 8 *a*, can be obtained by taking the barium salt precipitated by 30–50 % ethanol, dissolving it in water and reprecipitating with 30 % ethanol.

10. The fractions 8 *a* and 9 are combined, precipitated with lead acetate and the lead salts decomposed in N/10 H_2SO_4. This final stage of the purification depends upon the precipitation of various impurities as their lead salts at a pH lower than that at which the lead salt of the coenzyme precipitates (cf. Table 4). The separation is conveniently done as follows: saturated lead acetate solution is added drop by drop to the solution of the crude coenzyme in N/10 H_2SO_4 until a turbidity forms; 3–4 more drops are added and the precipitate is left to coagulate before centrifuging down; another 3–4 drops of lead acetate are then added and the process is repeated until the addition of a drop of lead acetate produces no further precipitate. In this way the bulk of the coloured material is precipitated together with one-third to one-half of the coenzyme; this slow fractional precipitation is essential to avoid adsorption of most of the coenzyme on the coloured material. The supernatant remaining is coloured faintly yellow. N/10 NaOH is now added drop by drop until the pH reaches 6·8–6·9 and the precipitate left for 30 min. to coagulate. This precipitate is white and represents the purest preparation of codecarboxylase so far obtained. A further yield can usually be obtained by decomposing the lead salts of the coloured material obtained in the early part of this stage and repeating the whole process of fractional lead salt precipitation under the same conditions.

The yield of material obtained in stage 10 represents 3–5 % of that originally present in the dried yeast, and the purification is 12,000–15,000 times. The fractions are best kept as their lead salts, in which state they can be dried *in vacuo* and kept indefinitely, being regenerated for use by decomposition in N/10 H_2SO_4. The yield of lead salt from fraction 10 is approx. 10 mg./kg. of dried yeast extracted. This fraction, freed from lead, gives a

Table 5. *Purification of codecarboxylase from dried brewer's yeast*

	Apo-enzyme ...		Lysine decarboxylase				Tyrosine decarboxylase		
Stage	Preparation	Total vol. (ml.)	Units of activity/ ml.	Total units	mg. C/ ml.	P_{Lysine}	Units of activity/ ml.	Total units	$P_{Tyrosine}$
1	Yeast-baryta-suspension	10,500	13·3	140,000	38·0	420	3·3	34,000	104
	Supernatant	8,300	8·85	73,500	11·5	925	2·85	23,700	298
2	Ba precipitate decomposed	620	112	69,000	4·24	31,700	34	17,000	9,620
3	First phenol extract (discarded)	84	90	7,560	3·13	34,500	23	1,930	8,820
4	Second phenol extract	128	220	28,000	2·45	108,000	49	6,250	24,000
5	*iso*Butanol extract	138	140	18,000	0·58	284,000	32	4,400	66,000
6	First Pb salt (decomposition)	66	265	17,500	0·789	404,000	64·5	4,250	98,100
7	SO_4 removed with Ba	68	220	15,000	—	—	58	3,950	—
8	Ethanol fraction: Ba salt								
	(*a*) 0–30 % ethanol	12	515	6,200	0·346	1,800,000	175	2,100	608,000
	(*b*) 30–50 % ethanol	8	600	4,800	0·688	1,050,000	190	1,500	332,000
	(*c*) 50–70 % ethanol	5	140	700	0·475	355,000	31	205	104,000
9	0–30 % ethanol fraction of 8 *b*	4	620	2,480	0·46	1,620,000	210	780	550,000
	8 *a* plus 9	16	—	8,680	—	—	—	2,880	—
10	Final Pb fraction at pH 6·8	10	420	4,200	0·100	5,050,000	130	1,300	1,560,000

colourless or faintly yellow solution. The figures quoted for the recombination of the specific proteins of lysine and tyrosine decarboxylases with codecarboxylase refer to preparations of the latter corresponding to fraction 10 (Gale & Epps, 1944; Epps, 1944).

Ultra-violet absorption spectra. Fig. 1 shows the ultra-violet absorption spectra of codecarboxylase solutions containing (1) 0·052 mg. C/ml. (neutral) and (2) 0·058 mg. C/ml. in N/10 H_2SO_4. There is an absorption band with a peak at 265mμ but no marked absorption in the 350–450mμ range, so that the substance does not appear to be a flavin derivative. Reduction of the substance with cysteine or hydrosulphite does not alter the shape of the absorption curve significantly.

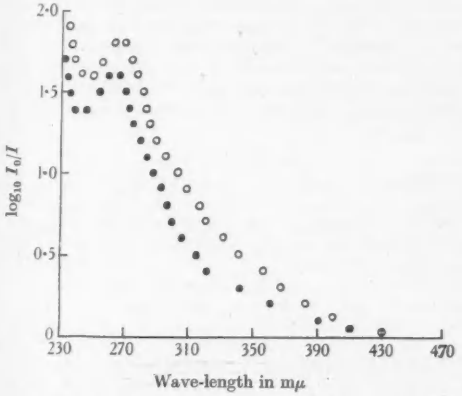

Fig. 1. Ultra-violet absorption spectra ($d=1$). ○ Solution containing 58 μg. C/ml. in 0·1 N-H_2SO_4. ● Solution containing 52 μg. C/ml., neutral.

Empirical composition. Analysis of lead salt of fraction 10 (Weiler): C, 16·00; H, 2·31; N, 3·59; Pb, 69·8%; P, nil; S, nil.

Stability towards acid and alkali. Suitable amounts of the codecarboxylase, freed from lead, were dissolved in N/2 and N/10 NaOH and H_2SO_4 respectively and placed in a boiling water-bath. Samples were removed at intervals for test of codecarboxylase activity. Table 6 shows that the activity is not affected by 4 hr. at 100° in N/2 or N/10 NaOH. In N/10 acid at this temperature the activity is rapidly destroyed.

Table 6. *Stability to acid and alkali of codecarboxylase preparations*

(Activities expressed in units of codecarboxylase/ml. before and after treatment with acid or alkali.)

Time at 100° (min.)	N/10 H_2SO_4		N/10 NaOH	
	Lysine decarb-oxylase	Tyrosine decarb-oxylase	Lysine decarb-oxylase	Tyrosine decarb-oxylase
0	380	31·5	380	31·5
30	207	22·1	—	—
60	110	11·3	—	—
90	66	6·6	—	—
120	22	2·0	—	—
240	—	—	365	32·1

Stability towards nitrous acid, etc. Solutions of codecarboxylase left to stand with HNO_2 or HOBr, or shaken in alkali with benzoyl chloride, followed by removal of the reagents, show no decrease of coenzyme activity.

Identity of the coenzymes of lysine and tyrosine decarboxylases. Whenever possible, preparations have been assayed for coenzyme activity with both lysine and tyrosine apo-decarboxylases. It can be seen from Table 1 that the distribution of the coenzyme amongst various tissues is the same whether the assay is carried out with the lysine or the tyrosine apo-enzyme. Table 5 shows that the stages of the purification are the same for lysine codecarboxylase as for tyrosine codecarboxylase and that, for any one state, the purification achieved in one case is parallel to that obtained in the other. Table 6 shows that the stability of the coenzyme to hydrolysis is the same whichever apo-enzyme is used for assay. It would appear probable from these sets of data that the codecarboxylase is identical for the two enzyme systems studied. If this be so, then it should be possible to activate the apo-enzyme of one with the coenzyme moiety of the other. Table 7 shows the results of an experiment in which this has been accomplished. Preparations were made of *l*(+)-lysine decarboxylase (Gale & Epps, 1944) from *Bact. cadaveris* and purified to the highest degree so far obtained ($Q^c_{CO_2}=33{,}500$) and of *l*(−)-tyrosine

Table 7. *Activation of tyrosine apo-decarboxylase by coenzyme moiety of lysine decarboxylase*

Manometer no. ...	1	2	3	4	5	6	7
	Additions (ml.)						
M/5 Acetate pH 5·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5
M/15 Tyrosine	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Tyrosine apo-decarboxylase	0·5	0·5	0·5	0·5	—	—	—
Codecarboxylase (excess)	—	0·5	—	—	0·5	—	—
Boiled tyrosine decarboxylase preparation (8 units)	—	—	0·5	—	—	0·5	—
Boiled lysine decarboxylase preparation (15 units)	—	—	—	0·5	—	—	0·5
Water	0·5	—	—	—	—	—	—
μl. CO_2/5 min.	15	227	156	160	0	3	0

decarboxylase from *Strep. faecalis* (Epps, 1944). The specific protein of the tyrosine decarboxylase was then precipitated and freed from codecarboxylase (Epps, 1944). The activity of this specific protein was then tested towards tyrosine alone and in the presence of codecarboxylase (preparation 8*a*); a boiled preparation of tyrosine decarboxylase (8 units), and a boiled preparation of lysine decarboxylase (15 units). The values in Table 7 show that the apo-enzyme can be reactivated towards its substrate by the presence of the codecarboxylase preparation, or of the boiled inactive preparations of either enzyme. Thus the lysine decarboxylase preparation contains a substance which will act as the codecarboxylase of the tyrosine decarboxylase.

SUMMARY

1. Codecarboxylase is widely distributed amongst animal tissues, plant tissues, yeasts and bacteria.

2. Codecarboxylase can be extracted from dried brewer's yeast by mildly alkaline solutions.

3. A method is described for the purification of codecarboxylase extracted from dried brewer's yeast. An over-all purification of 15,000 times can be achieved.

3. The resulting preparation is colourless or faintly yellow, contains C, H, N but no P or S, and has a single absorption band in the ultra-violet at 265mμ.

4. The coenzyme activity is stable to alkaline hydrolysis but is rapidly destroyed by N/10 H_2SO_4 at 100°.

5. Evidence is presented that *l*(+)-lysine decarboxylase and *l*(−)-tyrosine decarboxylase have the same codecarboxylase.

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A Liver Storage Test for the Assessment of Vitamin A

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The introduction of chemical methods for the determination of vitamins led most investigators to prefer these methods to biological assays. The chemical methods are simpler, cheaper and require less time. The physiologist, however, is not only interested in the quantity of vitamin given, but in its utilization by the organism. This question can be answered by the biological assay. Often, especially in the investigation of liver oils, there are considerable differences among the chemical, physical and biological results of vitamin A determinations (Coward, Dyer, Morton & Gaddum, 1931; Edisbury, Gillam, Heilbron & Morton, 1932; Morgan, Edisbury & Morton, 1935; Pritchard, Wilkinson, Edisbury & Morton, 1937; Robinson, 1938; Embree, 1939; Lunde & Kringstad, 1940). These differences have brought about such uncertainty regarding the value of the vitamin A potency of some materials that in these cases it is now agreed to consider only the results of bioassay as reliable. Nevertheless, great disadvantages of the methods of bioassay for vitamin A lie in the time and effort needed for their performance; and this is especially true of the curative growth test, the method most commonly used.

The difficulties in the determination of the vitamin A content of biological materials are even greater, if these materials contain both vitamin A and carotene, or even carotene alone. The usual chemical method for carotene determination, that of Guilbert (1934), is not exact enough, because α-, β-, and γ-carotene and kryptoxanthin are all determined together, and although all are precursors of vitamin A they differ in their biological potency. Because of the different amounts of the various carotenes present in different plant foods, this kind of determination cannot give the real vitamin A

potency of the substance tested. At present no adequate means exist which allow quantitative separation of the individual carotenoids. Although admirable in the qualitative separation of carotenoids, the technique of chromatography has yet to prove of value in their quantitative determination (Peterson, 1941).

These considerations led us to seek a shorter and simpler biological method than those in use. Our starting point was the fact that the liver is the most important reservoir of the body's vitamin A (Moore, 1931; Baumann, Riising & Steenbock, 1934). Consequently we considered the possibility of determining the available amount of vitamin A in materials by feeding them to rats and then determining chemically the amount of vitamin A stored in the liver. The results of such a method would depend upon the utilization of vitamin A by the organism and would be obtained within a few days. Before accepting such a method of bioassay it would be necessary to decide whether there exists a constant relation between the amount of vitamin A given to the rats and the amount stored in their livers.

METHODS

Rats weighing 35–50 g. were used. The food of the mothers consisted of sprouted wheat, barley, bran, the seasonal vegetables and milk. Two weeks before their progeny were weaned no vegetables were included in their food, the amount of vitamin A thus being reduced. The livers of the young rats then contained only 3–10 i.u. vitamin A. This amount of vitamin A disappeared completely within 2–6 days, when the young rats were given a vitamin A-free diet consisting of 65% rice flour, 13% casein (ethanol extracted), 10% olive oil, 8% dried yeast, 4% salt mixture and vitamin D, 100 i.u./kg. food (mixed with the oil).

This treatment produced rats with no signs of avitaminosis, which were suitable for our experiments.

Vitamin A ('Prepalin', Glaxo Laboratories Ltd.) was administered *per os* in 0·1 ml. olive oil. On the first and second days the rats were given a known amount of vitamin A/10 g. body weight. On the fourth day the rats were guillotined, the livers prepared according to Lindquist (1938), and their content of vitamin A determined according to the method of Carr & Price. The estimation was made by means of a block-comparator. The comparison colour was a solution containing 6·5% $CuCl_2$ and 0·3% $CoNO_3$ (Kuhn & Brockmann, 1931). For the control colorimetric determination vitamin A ('Prepalin') was used as a standard.

Some authors prefer the stufenphotometer or the photoelectric colorimeter, because they give a more exact determination of the rapidly fading blue colour. We found, however, that with a little experience with the block comparator we could obtain a high degree of precision. Control estimations showed that the experimental error never exceeded 10%. Pett & Le Page (1940) also report a satisfactory degree of accuracy with a simple colorimeter.

Altogether the livers of 312 rats were examined, 156 males and 156 females. The rats were divided into five groups, each rat of each group receiving on the first and second day of experiment 10, 15, 20, 30 or 35 i.u. of vitamin A/10 g. body weight respectively. Each group contained the same number of males and females. For the examination two livers from rats of the same group and the same sex were pooled.

RESULTS AND DISCUSSION

After the administration of various amounts of vitamin A to rats depleted under conditions as specified above, the amounts of vitamin A shown in Table 1 were recovered from the liver.

Table 1. *Amounts of vitamin A recovered from rat liver after its administration*

No. of pairs of rats*	Vitamin A administered (i.u./10 g. body weight†)	Total vitamin A recovered (i.u.)	
		Mean	S.D.‡
42	10	4·3	0·905
24	15	10·8	2·18
56	20	16·4	4·02
24	30	24·5	3·23
10	35	29·5	2·69

* Equal numbers of males and females were used.
† The same dose was given daily for 2 days.
‡ S.D. = standard deviation.

A plot of the amounts administered on a logarithmic scale and the amounts recovered on a linear scale, as is done in Coward's reference line for the growth test (Coward, 1939, p. 34), gave not a straight line but a parabola (Fig. 1). The equation of this curve was (Toft & McKay, 1940, p. 279):

$$x = 0{\cdot}0601y^{0{\cdot}755} + 0{\cdot}8175,$$

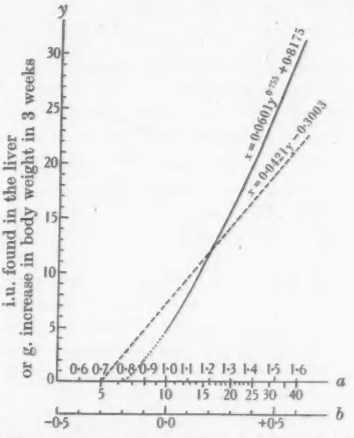

Fig. 1. Relation of amount of vitamin A stored in liver of rats to the dose administered. —— Liver storage reference curve. ----- Growth response reference curve of Coward.

(*a*) log units / i.u. } given/10 g. body weight.

(*b*) log units given (growth test).

where x = logarithm of vitamin A given expressed as i.u./10 g. body weight, and y = the total units recovered from the liver. This reference curve holds good up to 35 i.u. administered. The lowest amount actually administered was 10 i.u./10 g. rat; by inserting zero for y, it seems probable that 6·5 i.u./10 g. body weight will not cause any liver storage at all.

To compare the reliability of our method with that of Coward, three sets of growth tests were performed. As described by Coward (1939), young rats were kept on a vitamin A-free diet until no further increase in weight occurred. Then each of them was given 1 i.u. vitamin A/day for 3 weeks. Since the livers of two animals had to be pooled to obtain clear readings in the Carr-Price reaction in low concentrations, the mean increase in weight of two animals, paired at random, had to be taken for comparison. The results of these tests are given in Table 2.

Table 2. *Three weeks' curative growth test*

(Each rat was given 1 i.u. vitamin A daily for 3 weeks.)

Date	No. of pairs of		Weight increase (g./pair)	
	Males	Females	Mean	S.D.
July–Aug. 1941	3	3	9·0	3·64
Nov.–Dec. 1941	3	3	18·2	4·21
Oct.–Nov. 1942	6	6	15·2	4·88

To facilitate comparison, Coward's growth response line (3 weeks' test, equal numbers of males and females) has been superimposed on our curve in Fig. 1. The formula of this line is:

$$y = 23{\cdot}77x + 7{\cdot}14 \quad \text{or} \quad x = 0{\cdot}0421y - 0{\cdot}3003,$$

where x = logarithm of dose, and y = body-weight increase in g. Three methods were adopted to assess and compare the accuracy of the growth test with the liver storage test.

(1) According to Gaddum (1931, quoted by Coward, 1939, p. 165), a measure of the accuracy of the growth test is given by the S.D. (standard deviation) divided by the slope of the curve of response. This ratio, called the S.D. of the logarithms of the result, is denoted by λ. Since Coward's logarithmic reference line for the growth test is a straight line, the slope is at all points the same. In Fig. 1 the slope of the Coward line is 1·187. The S.D. of weight increase in our three sets of growth tests (24 pairs of animals) was found to be 4·27, whence λ equals 3·59.

Our data are not precisely comparable with those of Coward because, as already mentioned, we considered the S.D. of the weight increase of pairs, while Coward calculated it for single animals. In the 3 weeks' growth test Coward (1939, p. 171) found a S.D. of the weight increase for males ($\sigma\,♂$) of 11·86 g. and for females ($\sigma\,♀$) of 9·74 g. Therefore the S.D. of the weight increase for an equal number of males and females is:

$$\sqrt{\frac{\sigma♂^2 + \sigma♀^2}{2}} = 10{\cdot}85\,\text{g}.$$

The S.D. of the weight increase for an equal number of pairs of males and females can be derived without great error, if the S.D. of the weight increase of single animals is divided by $\sqrt{2}$:

$$\frac{10{\cdot}85}{\sqrt{2}} = 7{\cdot}68\,\text{g}.;$$

therefore

$$\lambda = \frac{7{\cdot}68}{1{\cdot}187} = 6{\cdot}47.$$

Our reference curve is a parabola. The slope, therefore, has to be determined by differentiation. Below are listed the values of λ for varying amounts of vitamin A administered/10 g. of rat:

I.u. administered	λ
10	0·573
15	1·105
20	1·860
30	1·357

All the values of λ are better than 3·59, which was obtained with the growth tests. The ratio of the values obtained from liver storage and growth tests is 1·86 : 3·59, even when we take the '20' level, which has the highest value of λ. Furthermore, from the standard errors of the two tests it may be calculated that $3\frac{1}{2}$ times as many rats would have to be used in the growth test to obtain the accuracy of the liver storage test.

(2) The probable error of a growth test is estimated to be 20 % in the 3 weeks' growth test, and about 16 % in the 5 weeks' growth test, when ten male rats are used. In our three growth tests, based on 24 pairs of animals only, the probable error was 12 %.

In the liver storage test, even if we take the less favourable results obtained at a level of 20 units administered, the probable error was only 6·5 %, with five pairs of animals. This agrees with the results of calculations given above; for if one takes 3·7 times as many pairs of animals, i.e. 17 pairs instead of 5, the probable error becomes 6·5 % instead of 12 %.

(3) As a check on the calculations, a further series of tests were made. Groups of rats, each consisting of two pairs, were given a varying number of units of vitamin A. In the first series, different groups were given 10, 20 and 35 i.u./10 g. body weight. The liver storage of vitamin A in the first group amounted to 2·75 i.u., in the second 10·25 i.u., and in the third 21·0 i.u. (Table 3). From our reference curve 2·75 i.u. recovered corresponds to 8·8 i.u. given; in other words the 10 i.u. administered acted only as 8·8 i.u., or the factor of potency of the

material used in relation to our curve is 8·8 : 10 = 0·88. Similarly for the dose of 20 units a factor of potency of 0·74 was obtained, and for 35 units, 0·76. Like Coward's reference line for the growth test, our curve does not give absolute but only relative values. Hence if we take the 20 units level, which lies roughly in the middle of our range, to be 100 %, the 10 i.u., in comparison with the action of 20 i.u., will be as 0·88 : 0·74 = 119 %, and the 35 i.u. as 0·76 : 0·74 = 103 %. The relative deviation would thus be 19 % in the first and 3 % in the second case. Further sets of tests are listed in Table 3.

In the results tabulated, which admittedly represent rather few observations, the mean percentage error is 9·5, when rats receiving 20 i.u. are used as a base and other suitable amounts are given to other pairs. In these tests the S.D. of the experimental errors is 11·8 %, and the probable error is 7·8 %. Assuming a difference of twice the S.D. as probably not being due to chance, a deviation of more than 24 % from the base would be significant; none of the experiments showed a deviation of more than 19 %.

On the basis of the reasons given, it seems to us that the liver storage test has an accuracy at least of the same order as the 3 weeks' curative growth test, and has the advantage of being much shorter. Its limitations lie in the fact that the materials to be tested must contain an amount of vitamin A which will cause appreciable storage of vitamin A in the liver, when administered on two successive days after depletion of the normal stores in the liver.

Table 3. *Relative deviations of the amounts of vitamin A recovered from rat livers after administration of varying doses*

No. of pairs of rats	Vitamin A (i.u.) Given	Vitamin A (i.u.) Recovered from liver	Value of amount given* (i.u.)	Factor of potency†	Value of amount given (% of 20 i.u.)	Deviation from 20 i.u. value (%)
2	10	2·75	8·8	0·88	119	19
2	20	10·25	14·7	0·74	100	—
2	35	21·00	26·6	0·76	103	3
2	10	3·35	9·3	0·93	102	2
2	20	14·00	18·2	0·91	100	—
2	35	24·35	31·7	0·91	100	0
2	10	3·70	9·5	0·95	117	17
2	20	11·75	16·2	0·81	100	—
2	35	25·00	32·8	0·94	116	16
2	10	2·70	8·8	0·88	109	9
2	20	11·80	16·2	0·81	100	—
2	10	3·80	9·6	0·96	99	1
2	20	15·00	19·3	0·97	100	—
2	10	3·70	9·5	0·95	83	17
2	15	11·10	15·6	1·04	90	10
2	20	18·50	23·0	1·15	100	—
2	30	27·00	35·7	1·19	103	3
2	10	4·10	9·8	0·98	82	18
2	15	13·70	18·0	1·20	100	0
2	20	19·10	24·0	1·20	100	—
2	30	27·00	43·0	1·43	119	19
2	8	0	>6·5	>0·8	—	—
2	12	0·90	7·4	0·62	103	3
2	16	2·10	8·3	0·52	87	13
2	20	7·00	11·9	0·60	100	—
2	24	9·20	14·0	0·58	97	3
2	28	13·60	17·9	0·64	107	7
2	32	11·00	15·7	0·49	82	18

* Obtained from liver storage reference curve.

† Factor of potency is: $\frac{\text{Value of amount given}}{\text{Actual amount given}}$.

SUMMARY

1. A method for the bioassay of vitamin A is described, which is based on the determination of the amount of vitamin A stored in the livers of rats previously depleted of this vitamin, when the material under test is fed on two successive days.

2. This test seems to be no less accurate than the 3 weeks' curative growth test, and requires only 4 days to perform.

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The Biological Value of Carotene from Various Sources and the Effect of Vitamin E on the Utilization of Carotene and of Vitamin A

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In 1934 the Second International Conference on Vitamin Standardization defined the international unit of vitamin A as the vitamin A potency of 0·0006 mg. of β-carotene. In the last decade, however, it has become clear that in many cases a simple relationship does not agree with the findings. In experiments with human beings (Booher & Callison, 1939; Booher, Callison & Hewston, 1939) and with animals (Guilbert, Howell & Hart, 1940; Smith & Otis, 1941; Treichler, Kemmerer & Fraps, 1942) it was found that vitamin A was utilized better than carotene. Moreover, several authors (Dyer, Key & Coward, 1934; Kraybill & Shrewsbury, 1936; Lease, Lease, Steenbock & Baumann, 1939; Sherman, 1941; Treichler *et al.* 1942) pointed out that the utilization of carotene by rats was influenced by the nature of the oil used as solvent. It appears, therefore, that the resorption and transformation of carotene into vitamin A depends on factors of which the nature and mechanism are as yet unknown.

It seems, therefore, that existing evidence is contrary to the assumption that 0·0006 mg. β-carotene always has the biological potency of 1 i.u. vitamin A. Its efficiency as pro-vitamin A depends, apparently, on many factors intimately connected with the material in which it is contained (various oils, plant materials). A more suitable descriptive term of the quantitative biological potency of carotene would be the *biological value of carotene*, which may be defined as:

$$\frac{\text{Actual vitamin A potency of the material, expressed in terms of i.u.}}{\text{Carotene content of the material, determined chemically, expressed in terms of i.u.}} \times 100.$$

The liver storage test, described in a previous communication (Guggenheim & Koch, 1944), served as a useful means for assessing vitamin A potency. Using this method, we have determined the biological value of carotene of various human and animal foods.

METHODS

For each examination, 8 or 10 test rats and an equal number of control rats were used, each group comprising an equal number of males and females. The preparation of the rats for the experiments, the vitamin A-free diet, the method of vitamin A determination in the rat livers, and the experimental procedure have been described in a previous paper (Guggenheim & Koch, 1944). The test rats and the control rats were given on two successive days the material to be tested or a measured amount of vitamin A respectively. On the fourth day the rats were killed and their livers examined. In the present study 'Prepalin' (Glaxo Laboratories Ltd.) was also used as a vitamin A standard preparation. The respective amounts of carotene and vitamin A of the materials given to the test rats, and that of vitamin A given to the control animals, were chosen according to their body weight. Carotene was determined according to the

method of Guilbert (1934) as modified by Peterson, Hughes & Freeman (1937).

The method of determining the biological value of carotene is illustrated by this example. A sample of carrots which contained 7·5 mg. of carotene/100 g. was tested on 10 test rats and 10 control rats, weighing 42–49 g.; the average weight of each group was 46 g. The control rats received 46 i.u. vitamin A each, i.e. 10 i.u./10 g. body weight, on two successive days; the test animals were given 1 g. of carrots = 0·075 mg. carotene, assumed to be equal to 125 i.u., or 27·2 i.u./10 g. body weight. The average amount of vitamin A recovered from the livers of the control rats was 6·0 i.u., and that from the test animals, 13·2 i.u. According to our curve (see Guggenheim & Koch, 1944), 6·0 i.u. recovered correspond to 11·2 i.u. given, i.e. in this experiment the reaction of the control rats was the same as if they had been given 11·2 i.u./10 g. body weight instead of 10 i.u. The 13·2 i.u. recovered from the livers of the test rats which received carotene correspond on our curve to 17·4 i.u. administered. From the equation

$$\frac{x}{10}=\frac{17{\cdot}4}{11{\cdot}2}, \text{ or } x=15{\cdot}5,$$

where x equals number of i.u. given to the test rats/10 g. body weight, and 10 equals the number of i.u./10 g. body weight of vitamin A given to the control rats, we find that the vitamin A potency of the carotene contained in the carrots fed to the test rats is 15·5 i.u./10 g. body weight. The amount of carotene determined chemically was, however, 27·2 i.u.; hence the biological value of carrot carotene is

$$100\times\frac{15{\cdot}5}{27{\cdot}2}=57.$$

RESULTS

The biological values of plant materials and their carotenoid content. Table 1 presents the biological values of vitamin A and carotene in the various foodstuffs fed to the experimental rats. The biological value of the vitamin A preparations 'Navitol' (E. R. Squibb and Sons), which contains vitamin D, and of 'Nadola' (Parke Davis and Co.) which contains no vitamin D, is *c.* 100. A similar value was also obtained for vitamin A and carotene from cow's liver. It appears, therefore, that the utilization of carotene and vitamin A from these sources is complete, while that of carotene from plant material is less so and variable; the range of the biological values is 33–67. There is one exception in lettuce, the carotene of which is utilized as efficiently as is pure vitamin A.

What are the factors responsible for the variable utilization of carotene? The main factor is, no doubt, the composition of the carotene, i.e. the amount of β-carotene contained in the carotene mixture. Only few of the materials tested have as yet been thoroughly studied as to the forms of carotene they contain, e.g. carotene of leaf (Mackinney, 1935), spinach (Karrer & Schlientz, 1934; Beadle & Zscheile, 1942), carrots (Kuhn & Lederer, quoted by Bredereck, 1936), apricots (Brockmann, 1933) and maize (Sherman, 1940; Fraps & Kemmerer, 1941), but since the amount of pro-vitamin A found by chemical analysis is very much larger than the biological value, we must conclude, therefore, that, apart from the effect of the varying carotene mixture of different pro-vitamin A activity, there exists a factor (or factors), which influences the utilization of carotene by the organism.

Faecal excretion of carotene. One such factor may be faulty resorption from the intestine. Hence we studied carotene excretion in the faeces. Preliminary tests yielded the following results. (1) The

Table 1. *Biological values of carotenoids in various foodstuffs*

$$\text{Biological value}=\frac{\text{Vitamin A potency of material in i.u.}}{\text{Carotene content of material in i.u.}}\times 100.$$

No. of test rats	Source	Kind of carotenoid	Biological value
10	'Nadola'*	Vitamin A	97
10	'Navitol'†	Vitamin A	100
10	Beef liver	,, +carotene	100
10	,,	,, +carotene	101
10	Lettuce	Carotene	105
10	,,	,,	102
8	,,	,,	96
8	Maize leaves	,,	67
8	,,	,,	66
10	Spinach	,,	67
8	,,	,,	62
10	Carrots, raw	,,	63
10	,, ,,	,,	60
8	,, ,,	,,	57
8	,, ,,	,,	57
10	Carrots, cooked	,,	62
8	,, ,,	,,	54
10	Apricot	,,	63
8	,,	,,	50
8	Dried grass	,,	58
8	,,	,,	55
10	Tomato juice, fresh, concentrated	,,	54
10	,, ,,	,,	49
10	Tomato juice, canned, concentrated	,,	42
10	,, ,,	,,	39
10	,, ,,	,,	37
10	,, ,,	,,	34
10	Savoy cabbage	,,	48
8	,,	,,	46
10	Carotene in olive oil‡	,,	46
10	,, ,,	,,	40
8	,, ,,	,,	47
8	,, ,,	,,	43
10	Pumpkin	,,	49
10	Maize flour	,,	39
10	,,	,,	37
10	Alfalfa	,,	39
10	,,	,,	33

* Vitamin A preparation (Parke Davis and Co.), contains 50,000 i.u. vitamin A/ml.

† Vitamin A preparation (E. R. Squibb and Sons), contains 50,000 i.u. vitamin A and 10,000 i.u. vitamin D/ml.

‡ Carotene (S.M.A. Corporation), contains 90% β-carotene and 10% α-carotene.

Table 2. *Excretion of carotene after feeding various amounts in foodstuffs*

No. of rats	Source of carotene	Amount of carotene given daily for 2 days (mg./rat)	Excretion of carotene	
			Total (mg.)	% of intake
5	Lettuce	0·078	0·106	68
8	,,	0·033	0·044	66
5	Carrots	0·051	0·051	50
5	,,	0·102	0·102	50
5	,,	0·204	0·183	45
5	Maize flour	0·015	0·006	20
5	,,	0·050	0·019	19
5	Carotene in olive oil	0·035	0·014	20
10	,,	0·050	0·019	19
8	,,	0·080	0·028	18

faeces of rats not receiving carotene contained a pigment similar to carotene, which has already been described by several authors (Kemmerer & Fraps, 1938, Ramasarma & Hakim, 1942; Russel, Taylor, Walker & Polskin, 1942). The daily amount excreted in the faeces of the rat is equivalent to 0·5–2·0 μg. carotene. (2) Carotene excretion by animals receiving carotene by mouth ceases one day after the last dose has been fed. On the following day the faeces are practically free from carotene.

In the experiments on carotene excretion the same procedure was used as in those on the biological value of carotene. On the first and second day of the experiment the rats received a measured amount of carotene; the faeces of these two days and of the following day were collected and their combined carotene content determined. For control purposes varying amounts of carotene from various sources were fed to different groups of rats.

From the results (Table 2) it would seem that (1) the percentage of carotene excreted is independent of the amount fed; (2) the amount of carotene excreted is more or less constant for each source of carotene. There are, however, certain variations. We found, for instance, in 19 experiments on carrot carotene that the amount of carotene excreted varied between 39 and 60 % (average 49·9 %, S.D. 7·4 %); in 12 experiments with β-carotene in olive oil, 15–25 % was excreted (average 19·6 %, S.D. 4·4 %). A comparison of the amount excreted with the biological value of the carotene from the same material suggests that the biological value does not depend on the degree of excretion. On the contrary, the results show a correlation which appears paradoxical: a high biological value was found in cases of high faecal excretion and a low value in those of low excretion.

Effect of vitamin E on the utilization of vitamin A and carotene. Hickman, Harris & Woodside (1942) showed that vitamin E (α-tocopherol) improves the utilization of carotene and vitamin A in the curative growth test; they attribute this action of vitamin E to its anti-oxidative property, which protects vitamin A and carotene against oxidation. Their observations offer an explanation of our findings. (1) Protection against oxidation would account for the

Table 3. *Effect of α-tocopherol on the utilization and excretion of vitamin A and carotene*

No. of rats	Carotenoid given in 0·2 ml. olive oil on two successive days	α-tocopherol, amount given in 0·1 ml. olive oil together with the carotenoid (mg.)	Vitamin A found in liver (i.u./rat)	Biological value	Carotene excretion	
					Total (mg./rat)	% of intake
12	Vitamin A, 100 i.u.	0	18·5	100	—	—
8	,,	0·1	22·0	122	—	—
8	,,	0·5	25·0	144	—	—
8	,,	2·0	31·0	196	—	—
8	,,	10·0	45·0	*c.* 300	—	—
8	Carotene, 0·08 mg.	0	4·5	44	30	19
8	,,	0·1	6·5	47	38	24
8	,,	0·5	11·0	68	47	29
8	,,	2·0	15·0	84	55	36
8	,,	10·0	18·0	99	66	41

Table 4. *Effect of α-tocopherol on the utilization and excretion of carotene from carrots*

The control rats received 10 i.u. vitamin A/10 g. body weight on two successive days. 6·0 i.u. vitamin A were recovered from each liver. The test rats received 1 g. carrots, containing 0·075 mg. carotene = 125 i.u. = 27·2 i.u./10 g. body weight, on two successive days.

No. of rats	α-tocopherol, amount given in 0·1 ml. olive oil together with carrots (mg.)	Vitamin A found in liver (i.u./rat)	Biological value	Carotene excretion	
				Total (mg./rat)	% of intake
8	0	13·2	57	68	45
8	0·05	19·8	81	81	54
8	0·2	20·2	83	83	55
8	0·5	23·7	102	86	57
8	1·0	27·2	119	104	69

good utilization of carotene as well as for the high excretion. (2) The highest biological value of plant carotene among the materials tested was that for lettuce carotene, and lettuce, as is well known, is characterized by its high vitamin E content.

To test the hypothesis regarding the effect of vitamin E both on the utilization of vitamin A and of carotene, and on carotene excretion, we carried out three sets of experiments in which experimental rats received measured amounts of vitamin A, or carotene in olive oil, or carrots, together with varying quantities of α-tocopherol. The amounts of vitamin A stored in the liver, and of carotene excreted, were examined. The experimental details and the results are given in Tables 3 and 4.

The data show that the addition of α-tocopherol to vitamin A, or carotene in olive oil, or carrots, raises the biological value of vitamin A and carotene and increases the amount of carotene excreted.

This dependence of the utilization of vitamin A and of carotene on dietary vitamin E possibly also explains the individual effect of various plant oils. To test this possibility we chose six oils: olive, sesame, coconut, cotton-seed, peanut and linseed; the olive oil was refined, the others unrefined. According to Karrer & Keller (1938), the respective amounts of α-tocopherol in olive, coconut, sesame and linseed oils are 0·08, 0·03, 0·05, 0·23 mg./g. According to Scudi & Buhs (1942), unrefined cotton-seed oil contains 0·92 mg. α-tocopherol/g.

DISCUSSION

The results of our estimations of the biological value of carotene and vitamin A agree more or less with those obtained by other authors using other methods. Smith & Otis (1941) observed that the amount of vitamin A found in the livers of avitaminotic rats fed on carotene varies according to the source of the carotene. Booher & Callison (1939) and Booher *et al.* (1939), using the dark adaptation test, studied the utilization of carotene by humans and found that 0·0006 mg. carotene in cotton-seed oil is equivalent to no more than 0·55–0·6 i.u. vitamin A and that the carotene of peas was better utilized than that of spinach. The results of our feeding experiments with various oils also agree with those of other authors. Sherman (1941) studied the effect of various oils on the carotene utilization in curative growth tests. He found that carotene in cotton-seed or linseed oil was better utilized than in coconut oil. The excretion of carotene was 11·4–16·7 %. The results of Lease *et al.* (1939) differ somewhat from ours and from those of Sherman (1941), since they showed that carotene was better utilized when in coconut oil than in linseed oil. It is interesting to note that the refinement of peanut oil reduced its value as vehicle for carotene.

Davies & Moore (1941), the first to demonstrate a connexion in the metabolism of the vitamins A and E, found that addition of vitamin E to the diets of rats receiving vitamin A in cod-liver oil increased the amount of vitamin A stored in their livers. They also showed that the stores of vitamin A in the livers of rats on diets devoid of both vitamins A and E were more rapidly exhausted than those of rats receiving vitamin E. Bacharach (1940) confirmed these results. Hickman *et al.* (1942) demonstrated that vitamin E improved the vitamin A and carotene utilization in the curative growth test, and the latter authors showed, in *in vitro* experi-

Table 5. *Influence of various oils on the utilization and excretion of vitamin A and carotene*

No. of rats	Carotenoid given in 0·5 ml. of the oil on two successive days	Oil	Vitamin A found in liver (i.u./rat)	Biological value	Carotene excretion	
					Total (mg./rat)	% of intake
8	Vitamin A, 100 i.u.	Olive, refined	13·0	100	—	—
8	,,	Coconut, unrefined	15·0	112	—	—
8	,,	Sesame, unrefined	13·0	100	—	—
8	,,	Cotton-seed, unrefined	24·0	183	—	—
8	,,	Peanut, unrefined	18·0	132	—	—
8	,,	Linseed, unrefined	20·0	148	—	—
8	Carotene, 0·08 mg.	Olive, refined	4·3	44	27	17
8	,,	Coconut, unrefined	4·5	45	32	20
8	,,	Sesame, unrefined	4·2	44	39	24
8	,,	Cotton-seed, unrefined	12·2	72	78	49
8	,,	Peanut, unrefined	8·7	59	61	38
8	,,	Linseed, unrefined	9·3	61	67	42

In our experiments we dissolved appropriate amounts of vitamin A or carotene in the various oils. These oils, containing 200 i.u. vitamin A or 0·16 mg. carotene/ml., were fed to the test rats. The experimental details and the results are given in Table 5. For the calculation of the biological values we took that of vitamin A in olive oil as 100. From the results (Table 5) it appears that the different effects of the various oils correspond roughly with their relative content of vitamin E.

ments, that vitamin E protected carotene against oxidation. These results, which we have confirmed, indicate the mechanism of the tocopherol effect: by preventing oxidation in the intestine, α-tocopherol increases both resorption and excretion of carotene.

We think that the utilization of carotene contained in plant materials depends above all on their vitamin E content. The case of lettuce is very significant in this connexion. Of the vegetables, it contains the highest amount of vitamin E, namely, 0·55 mg./g. of dried lettuce (Karrer & Keller, 1938), and, correspondingly, the biological value and the amount of lettuce carotene excreted exceed those of all other plant materials examined by us. The results of our experiments with various oils point in a similar direction (Table 5).

It appears, therefore, that in the rat vitamin E plays an important role in the metabolism of carotene and vitamin A by improving their utilization. There is no reason to doubt that vitamin E exerts a similar action on the utilization of carotene and vitamin A by humans. Vitamin E is, therefore, for humans not a 'vitamin', i.e. a nutritional factor essential for maintenance and growth, but rather one which is important for the utilization of other nutrients. This also follows from the studies of Pavcek & Shull (1942), who observed that the oxidation and inactivation of biotin by rancid fats is inhibited by α-tocopherol.

The utilization of vitamin A and carotene depends, therefore, chiefly on the amount of vitamin E contained in the food. This fact necessitates a new scrutiny of the problem of human vitamin A requirement. Our present assumptions concerning this requirement do not take into account the vitamin E content of the food. The vitamin A requirement varies, probably, with the various vitamin E intakes, and a high amount of vitamin E in human diet will presumably decrease the vitamin A requirement.

SUMMARY

1. By the use of a liver storage test for the assessment of vitamin A, the utilization of vitamin A and of carotene from different sources was studied, and assessed in terms of biological values. The biological value of vitamin A contained in medical preparations and beef liver approaches 100; that of carotene in various plant materials ranges between 33 and 67, except in lettuce, in which it was found to be nearly 100.

2. The poor utilization of most of the plant carotenes is not caused by a large percentage of the less active or altogether inactive carotenoid pigments in these materials, or to a high carotene excretion with the faeces. The utilization of carotene from various plant sources, or of carotene dissolved in different oils, varies according to the vitamin E content of these materials.

3. Vitamin E increases both the utilization of vitamin A and of carotene, and the faecal excretion of carotene. Apparently it acts by protecting carotene and vitamin A against oxidation in the intestine, as a result of which their resorption and excretion are increased.

4. It is concluded that the human vitamin A requirement depends to a great extent on the vitamin E content of the food.

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The Urinary Elimination of Nicotinamide Methochloride by Man

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The development of a method for the estimation of nicotinamide methochloride in urine by Coulson, Ellinger & Holden (1944) provided a means of studying its quantitative elimination in human urine under a variety of conditions. A preliminary method (Coulson, Ellinger, Glock & Platt, 1942) which gave only comparative but not quantitative results had been used before the chemical nature of the pigments involved was elucidated by Huff & Perlzweig (1943), Ellinger & Coulson (1943) and Coulson & Ellinger (1943). This paper is concerned with the elimination of nicotinamide methochloride by a number of subjects under normal conditions and under the influence of some extrinsic factors. The effect of the intake of nicotinamide and several related compounds on the elimination of the methylated derivative was studied, and an attempt was made to determine the state of saturation in man with respect to nicotinamide.

EXPERIMENTAL METHODS

Method of determination of nicotinamide methochloride

Urine was collected at known intervals and the volume per hour was calculated. In the preliminary experiments 10 ml. were filtered through 1 g. columns of Decalso which were then washed with 50 ml. of water and eluted with 14 ml. of 25% (w/v) KCl. The eluates were divided into two equal parts, one of which was made alkaline with 1 ml. of 15% (w/v) NaOH. Both were shaken with 2 ml. of *iso*butanol for 5 min. The *iso*butanol extracts were transferred to small non-fluorescent test-tubes, dried with anhydrous Na_2SO_4 and their fluorescence intensity examined in the 366 mμ line of a high-pressure mercury lamp. The fluorescence intensity was given relative arbitrary units.

In the earlier experiments losses of nicotinamide methochloride occurred through incomplete adsorption due to high urinary electrolyte concentration, and the visual fluorimetry of the *iso*butanol extracts gave too low readings when the nicotinamide methochloride concentration was high. A conversion of these units into milligrams is, therefore, impossible. In the later experiments the quantitative method by Coulson *et al.* (1944) was used and the results were expressed in mg.

Collection of urines

Urine samples taken at frequent intervals throughout 24 hr. or complete pooled 24 hr. samples were collected from 24 subjects. The early experiments showed that the output of nicotinamide methochloride varied throughout the day. Consequently first morning samples were chosen, since these could be expected to be least affected by extrinsic factors such as food, alcohol in drinks, physical exercise, etc., during the period of collection. Seven-hour samples collected between 10 a.m. and 5 p.m. after ingestion of 100 mg. nicotinamide were later used for comparative experiments.

RESULTS

Normal daily elimination curves

Complete 24 hr. elimination curves were obtained from three subjects leading a uniform sedentary life, one normal male (1), one normal female (2) and one diabetic male (3) balanced by diet and insulin. The normal subjects were living on war-time diet, but the diabetic subject received increased rations of meat, butter, margarine, cheese and milk.

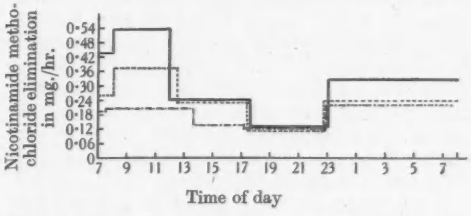

Fig. 1. Normal daily elimination curve of nicotinamide methochloride of subjects (1) ——, (2) - - - -, and (3) – – –.

The daily elimination of nicotinamide methochloride was also determined for 21 normal young airmen for 2 days by the earlier method. The results of the investigation of the three subjects is shown in Fig. 1. Each curve shown represents the average elimination on 3 normal days. The absolute daily output varies considerably.

Subject (1) eliminated an average of 7·6 mg., subject (2) 6·7 mg., and subject (3) 4·0 mg. The three curves, however, have the same shape, with a medium level in the early morning, a peak in the forenoon, a fall below the night level in the early afternoon, a decline to a minimum in the evening and a gradual rise during the night. The elimination curves gained from the 21 normal young airmen showed the same general shape.

There is some indication that the total daily output was subject to seasonal changes although the evidence at hand is not conclusive.

Effect of extrinsic factors

Food. As far as could be ascertained ordinary diet did not influence elimination of the methochloride. The morning rise is observed in fasting subjects as well as in those who had eaten breakfast. Liver in small amounts produced no significant increase in the output. The ingestion of 30 g. of dried yeast (*Torula utilis*, kindly supplied by Dr A. C. Thaysen) produced a slight increase.

Alcohol. Nicotinamide and related compounds are of value in the treatment of pellagra, and continuous and excessive intake of alcohol is associated with the onset of alcoholic pellagra; this suggested the study of the effect of ingestion of large amounts of alcohol in a short time on the elimination of nicotinamide methochloride following ingestion of nicotinamide or a related compound. For this purpose a fairly high output of nicotinamide methochloride was induced in subjects (1) and (3) by the daily intake of nikethamide in doses equivalent to 2·5–5·0 mg. nicotinamide/kg. body weight, and then 1 g. of alcohol/kg. body weight diluted with water to a 25 % solution was drunk in the space of 30 min. Neither subject was accustomed to large doses of concentrated alcoholic drinks. The daily nikethamide intake was continued for 4 weeks and alcohol was taken three times at intervals of about 10 days. In both subjects the alcohol imbibition was always followed by a temporary increase in the nicotinamide methochloride elimination and then by a rapid decline. This fall lasted 2–4 days after the first alcohol dosage, 3–6 days after the second, and for more than a week after the third.

Work. Observations on pellagrins in Yugoslavia and Egypt had shown that pellagrous symptoms were precipitated only in those engaged in hard work, while the rest of the population living on the same diet but leading a sedentary life showed only pre-pellagrous symptoms (Ellinger & Dojmi, 1935; and Ellinger, Hassan & Taha, 1937*a*); in addition, milder cases of pellagra were cured by rest alone without any change of diet after being admitted to hospital (Ellinger *et al.* 1937*b*). These observations suggested that work may play a considerable role in the metabolism of nicotinamide and consequently influence the urinary elimination of nicotinamide methochloride. Some indication that this might be the case was gained from the following experiment. Daily administration of small amounts of nicotinamide to the same subject produces a consistent rise of the same height and duration. One mg. of nicotinamide/kg. was taken by subject (3) each day before, during, and on the days after a 3-day period of unusually intense physical and mental strain. The elimination on the day before the strain was 450 arbitrary units; this decreased to 75 units on the first day after the strain, and the two following days showed a gradual rise to 321 units and 432 units respectively.

Effect of administration of nicotinamide and related compounds

The effect of a number of compounds on the elimination of nicotinamide methochloride has been studied. Nicotinamide, nicotinic acid, nikethamide and nicotinic acid-mono-ethyl-amide were found to be active, whereas trigonelline, pyridoxine, nicotinonitrile, aneurin or riboflavin did not increase the output of the methylated derivative.

The total elimination of nicotinamide methochloride for subjects (1), (2) and (3) after ingestion of various amounts of nicotinamide, nicotinic acid and nikethamide appears in Table 1.

Some indication of the rate of elimination was obtained by making determinations at 13, 37, 61 and 85 hr. after ingestion of nicotinamide, etc. The examination was discontinued when the amount eliminated fell to the average normal value. The data in Table 1 represent the mg. of nicotinamide methochloride eliminated, diminished by the average normal elimination during the same period of the day (based on a 3-day average (Fig. 1)). The figures in the last column for each subject are obtained by multiplying the mg. eliminated by 0·707 to correct for the difference in molecular weight between nicotinamide methochloride and nicotinamide.

The recovery of the methochloride after ingestion of nicotinamide from the normal subject (1) is two to three times greater than that from the diabetic subject (3); the recovery from subject (2) is more or less intermediate. Subject (1) eliminates about 25 % when given 100 mg. or more of the amide; if lower amounts are taken, the relative elimination is less. The same relation exists, although on a lower level, in subject (3); elimination by subject (2) is less consistent. During the first 13 hr. about 80 % of the total nicotinamide methochloride is eliminated irrespective of the amount ingested by all three subjects.

Compared with nicotinamide, nicotinic acid is converted to a smaller extent by subjects (1) and (2) (normals), the relative amount of the methyl-derivative eliminated by subject (3) being almost the same as after ingestion of nicotinamide. The conversion rate for nicotinic acid is much slower in all three subjects than for nicotinamide. The individual elimination curves of nicotinamide methochloride after intake of 100 mg. of nicotinamide and of nicotinic acid for subjects (1) and (3) are shown in Figs. 2 and 3.

After the ingestion of small doses of nikethamide the amount converted in subjects (1) and (2) is less than that for nicotinamide; after doses of 500 mg. the total amount converted is much the same as after similar amounts of nicotinamide. Subject (3)

Table 1. *Nicotinamide methochloride eliminated after ingestion of nicotinamide, nicotinic acid, and nikethamide by subjects (1), (2) and (3)*

Compound ingested	Dose (mg.)	Subject (1) Nicotinamide methochloride eliminated (mg.) during (hr.) 0–13	13–37	37–61	61–85	Total	Total (% of amount ingested)	Subject (2) Nicotinamide methochloride eliminated (mg.) during (hr.) 0–13	13–37	37–61	Total	Total (% of amount ingested)	Subject (3) Nicotinamide methochloride eliminated (mg.) during (hr.) 0–13	13–37	37–61	61–85	Total	Total (% of amount ingested)
Nicotinamide	50	6·8	—	—	—	6·8	9·6	—	—	—	—	—	3·0	—	—	—	3·0	4·2
	100	28·1	4·0	—	—	32·1	22·7	23·9	—	—	23·9	16·9	8·8	1·9	—	—	10·7	7·6
	200	72·0	5·4	—	—	77·4	27·4	44·2	—	—	44·2	15·6	24·3	0·7	—	—	25·0	8·8
	500	146·4	24·3	—	—	170·7	24·1	68·6	5·2	—	73·8	10·9	67·7	5·1	—	—	72·8	10·3
Nicotinic acid	100	13·4	—	—	—	13·4	9·5	2·9	6·2	—	9·1	8·2	6·4	1·8	—	—	8·2	5·8
	200	20·1	9·4	5·4	—	34·9	12·3	17·9	3·1	—	21·0	7·4	13·6	10·3	1·2	—	25·1	8·9
Nikethamide (in doses equiv. to nicotinamide on mol. wt. basis)	100	9·4	3·2	—	—	12·6	8·9	13·6	3·2	—	16·8	11·9	5·9	2·9	—	—	8·8	6·2
	200	19·8	18·0	—	—	37·8	13·4	18·5	8·7	—	27·2	9·6	13·2	11·1	3·3	—	27·6	9·8
	500	54·3	85·3	15·6	5·9	161·1	22·8	63·2	46·7	8·7	118·6	16·7	28·7	26·0	5·1	3·5	63·5	9·0

eliminates about the same percentage of the methylated derivative after ingestion of nicotinamide, nicotinic acid or nikethamide. In all three subjects the speed of elimination after ingestion of nikethamide is, however, considerably reduced and the duration prolonged; only 30–40 % of the total eliminated appears in the first 13 hr. Nicotinic acid-monoethyl-amide is similar in action to nikethamide.

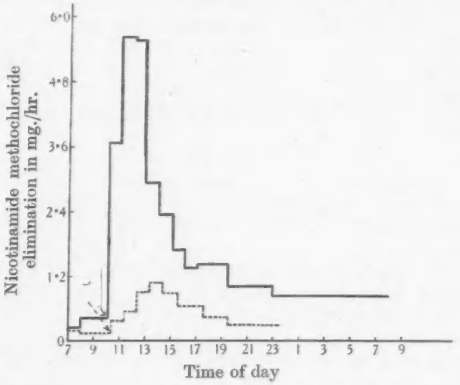

Fig. 2. Nicotinamide methochloride elimination after intake of 100 mg. of nicotinamide —— and nicotinic acid ----- by subject (1). Arrows indicate time of ingestion.

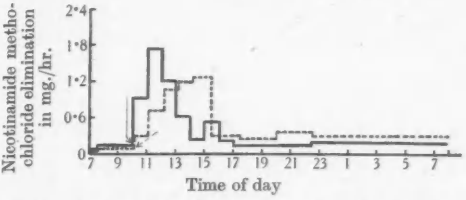

Fig. 3. Nicotinamide methochloride elimination after intake of 100 mg. of nicotinamide —— and nicotinic acid ----- by subject (3). Arrows indicate time of ingestion.

Fig. 4 shows a 2 days' average elimination curve of nicotinamide methochloride for 11 healthy young airmen after the intake of 100 mg. of nicotinamide. Although values used in the curve are in arbitrary units the curve is of the same shape as that shown in Fig. 2 for the absolute elimination in mg./hr. The maximum elimination in both curves occurs in the second hour.

The elimination of nicotinamide methochloride was also determined after oral and subcutaneous administration of nicotinamide methochloride in subjects (1) and (3). The recovery is given in Table 2.

The influence of continued daily administration of 100 mg. of nicotinamide on the nicotinamide

methochloride elimination was first studied by examining the first morning urine sample. Fig. 5 shows the nicotinamide methochloride elimination from two groups of young airmen (18–20 years)

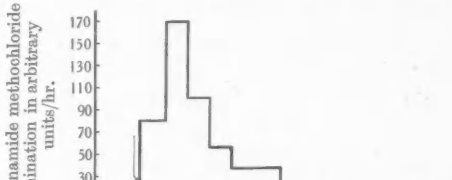

Fig. 4. Average nicotinamide methochloride elimination of 11 men for 2 days following the daily intake of 100 mg. nicotinamide. Arrow indicates time of ingestion.

living on the same diet and performing the same light physical work. Beginning with the fourth day of the experiment one group received daily a dose of 100 mg. for 7 days; the other group served as control. There is a marked but irregular rise of the

Table 2. *Recovery of nicotinamide methochloride after oral or subcutaneous administration in subjects* (1) *and* (3)

Dose (mg.)	Manner of administration	Recovery as % of intake during (hr.) 0–1	0–7	0–24
	Subject 1			
100	Oral	0·48	11·6	16·6
200	Oral	0·96	9·2	12·5
100	Subcutaneous	42·5	54·5	61·8
	Subject 3			
100	Oral	1·3	7·1	12·6
200	Oral	0·93	6·7	12·9
100	Subcutaneous	31·7	50·6	61·8

nicotinamide methochloride output; the level, however, is always higher than that of the controls. The depressions in the curves also occur in the control group and are probably due to extrinsic factors such as food, temperature, work, etc., equally affecting both groups, and therefore not connected with the administered nicotinamide.

Similar results were obtained from two other groups, each of 15 healthy young airmen. One of these groups received each day for 22 days mixed vitamin tablets containing 5 mg. of aneurin, 3 mg. of riboflavin, 50 mg. of nicotinamide, 5 mg. of pyridoxine, and 10 mg. of calcium pantothenate. Both groups were living on the same diet and working under the same conditions. The continued 'saturation' rise was interrupted on the fourteenth day of dosage for some unknown reason. A return to the former high level occurred a week later.

The effect of repeated ingestion of a daily dose of 100 mg. of nicotinamide on the elimination of the methyl derivative was studied in two groups of 10 and 8 laboratory workers selected at random and living on similar war rations. The group of 10 men

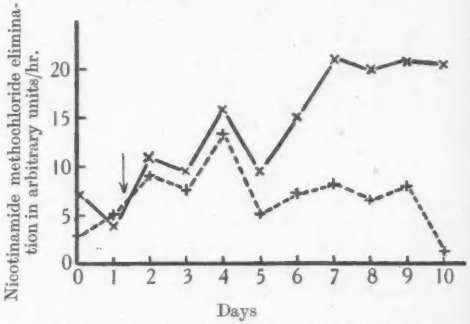

Fig. 5. Nicotinamide methochloride elimination in early morning samples of two groups of 10 men without +--+--+ and with ×——× daily intake of 100 mg. nicotinamide. The arrow indicates the beginning of the nicotinamide ingestion.

received 100 mg. of nicotinamide on the first day, no extra amide for the next 2 days, and 100 mg. on each of the following 4 days. The urine was collected during 7 hr. after the administration of the first, fourth and seventh days of the experiment and the nicotinamide methochloride content was determined. The group of eight men received 100 mg. nicotinamide a day for 5 consecutive days. The urine was collected for the 7 hr. intervals after the administration on the first, third and fifth days of the experiment and the methochloride estimated. The average elimination values in the first group were 13·6 mg. on the first, 14·9 on the fourth and 19·1 mg. on the seventh day. In the second group the values were 15·2 mg. on the first, 21·1 mg. on the third, and 22·3 mg. on the fifth day. These values have not been corrected by subtracting the normal daily excretion of the methyl derivative, which was unknown.

DISCUSSION

The shape of the daily normal curves seems to be consistent for the same individual at different times and indeed for different individuals. These fluctuations are still apparent on the second day after the ingestion of 500 mg. doses of nikethamide or nicotinamide, although the absolute height of the elimination is much greater. The relative height of the maxima in the normal curves varies considerably from day to day in the same individual and even more from person to person. The total daily elimina-

tion from our three subjects varied from 2 to 8 mg./day. These figures seem to be higher than those reported by Sarett (1943) for F_2 and by Wang & Kodicek (1943) for the total nicotinamide derivatives estimated in the urine. It seems probable that most of the so-called 'trigonelline' in the urine is nicotinamide methochloride, and that the trigonelline actually present is mainly due to that present in the diet. Trigonelline treated with alkaline *iso*-butanol does show a faint fluorescence, but the fluorescence efficiency in the 366 mμ line is so low as to be negligible in the assay. The ingestion of trigonelline showed no increase in the urinary output of nicotinamide methochloride.

It is not astonishing that the intake of the usual food has no immediate effect on the nicotinamide methochloride elimination, since the concentration of nicotinamide in the food now available is too low to produce a marked rise in the output. If the normal daily methochloride elimination is about 7·5 mg./day and if one assumes that approximately 15 % of the ingested nicotinamide is eliminated as the methyl derivative (the average elimination after nicotinamide doses), this would indicate the presence of more than 40 mg. of nicotinamide or nicotinic acid in the food ingested each day. This value is almost inconceivably high if one is to accept the values for the content of nicotinamide and similar compounds in the foods reported by numerous investigators. This suggests an extra dietary source of either nicotinamide, nicotinic acid or nicotinamide methochloride. It could be due to bacterial formation and the subsequent release of nicotinamide or nicotinic acid in the gut, as shown for aneurin by Najjar & Holt (1943) or less probably to the synthesis of one or all of these compounds in the human body. Experiments to study this question are in progress.

The effect of alcoholic drinks, a short increase in elimination followed by a lasting decrease, might be explained by the action of ethanol on the intestinal flora or by interference with the mechanism of methylation in the liver (Perlzweig, Bernheim & Bernheim, 1943). The amount of nicotinamide methochloride eliminated is probably influenced by physical exertion, and this must be taken into account in the evaluation of the normal daily output.

The rapid increase in the elimination of nicotinamide methochloride after the ingestion of nicotinamide shows that the methylation process is a rapid one. Only a small proportion of the ingested amide is eliminated as the methyl derivative; the highest proportion (43 %) occurred in subject (1) on the fifth day of a daily 100 mg. nicotinamide dosage. The fate of the remainder is not known. The daily increase in the output after successive doses of the active compounds and the relatively greater proportion eliminated after the higher doses than with small amounts seems to indicate a certain amount of storage in the body.

The elimination of nicotinamide methochloride depends not only on the amount of nicotinamide ingested but probably on the reserves of 'methyl-donators', i.e. substances that contribute methyl groups as well. On account of the present food restrictions it was impossible to examine this factor.

That still another factor is involved can be seen from the low conversion by the diabetic subject (3) who obtained far more animal protein and therefore more of the methyl donators than the others tested. This suggests a connexion between nicotinamide and carbohydrate metabolism, already described by Göbell (1941) and Neuwahl (1943), who found a reduction of the blood sugar after the intake of nicotinamide and nicotinic acid.

The conversion for subject (3) was much the same for nicotinamide and nicotinic acid, and showed that only the methylation but not the amidation was defective.

The results given in Table 2 show that nicotinamide methochloride is slowly and incompletely absorbed from the gut, and no other conclusion can be drawn from the experiments using oral administration. When given parenterally it is rapidly eliminated in the urine (about 40 % in 1 hr.); none can be found after 24 hr. About 40 % cannot be accounted for. The elimination of 61·8 % in 24 hr. by both subjects (1) and (3) shows that differences observed in the elimination of nicotinamide methochloride after the intake of nicotinamide by these two subjects cannot be due to different rates of destruction.

Pyridoxine had no effect on the elimination of nicotinamide methochloride; Single & Sydenstricker (1941) reported the elimination of another fluorescent substance after the ingestion of pyridoxine, which we were unable to confirm.

The physiological importance of the nicotinamide methochloride elimination in urine is not yet fully established. It is certain that the height of the elimination depends on the direct or indirect intake and on the storage of nicotinamide and related compounds, on the amounts of these compounds used up by the body, and probably on the amount of methyl-donators available in the body and on the efficiency of the methylating mechanism.

SUMMARY

1. The total daily urinary elimination of nicotinamide methochloride by man was determined and found to vary individually and in the same person at different times within the range of 2 and 8 mg. in 24 hr.

2. Ingested nicotinamide increases the elimination of nicotinamide methochloride. The study of the elimination suggests the occurrence of storage

and saturation of nicotinamide. Nicotinic acid, niketh amide and nicotinic acid-mono-ethyl-amide have a similar but quantitatively different action.

3. The hourly fluctuations of the nicotinamide methochloride elimination were studied and found to be identical in different persons and in the same person under different conditions.

4. The effect of a number of factors (food, alcohol, work) influencing the elimination of nicotinamide methochloride was studied.

5. The height of the nicotinamide methochloride elimination is determined by the intake of nicotinamide and related compounds and their use by the body, the presence of methyl-donators and the efficiency of the methylating mechanism.

6. The relation of nicotinamide methochloride eliminated in the urine to the nicotinamide ingested with the food indicates the presence of an extra dietary source of nicotinamide.

This work forms part of an investigation on nicotinamide deficiency carried out on behalf of the Air Ministry. We wish to thank Air Marshal Sir H. E. Whittingham, K.B.E., K.H.P., Director-General of the Medical Services of the Royal Air Force, for facilities provided, and Flight-Lieutenant G. A. Smart for collecting samples from airmen. Our thanks are also due to L.A.C.W. A. E. Wrigglesworth for technical assistance and to members of the Scientific and Technical Staff of the Lister Institute who volunteered as experimental subjects.

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Investigations on the Activity of the Histaminase in Normal and Toxaemic Pregnancy

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In some recent papers I have suggested that histaminase may play an important part in the metabolic changes occurring in normal and toxaemic pregnancy (Kapeller-Adler, 1941 *a*, *b*, *c*; Kapeller-Adler & Adler, 1943). From the experimental data obtained it has been assumed that histamine might be formed, in the metabolism of pregnant women, from the histidine present in large amounts throughout gestation, by the activity of histidine decarboxylase. In normal pregnancy most of the histamine formed is presumably destroyed by histaminase so that only traces escape destruction, to be excreted in the urine. It has further been suggested that in mild cases of pre-eclamptic toxaemia the activity of the histaminase may be impaired, and that more histamine may thus escape and be available to cause various kinds of damage. Much of it, however, seems to be eliminated in the urine. In severe cases of pre-eclamptic toxaemia and in eclampsia a condition may arise where the activity of the histidine decarboxylase may be increased, whereas that of the histaminase may be completely inhibited. Thus much histidine would be converted into histamine which, not being destroyed by histaminase, would cause considerable damage, especially to the liver and kidneys. The latter would then lose the ability to excrete histidine and histamine, which would be completely retained in the tissues. The object of the present paper was to obtain evidence of the role played by the histaminase in normal and toxaemic pregnancy.

Marcou (1937) reported that the blood in pregnancy has an extraordinarily high activity in destroying histamine. Other investigators (Werle &

Effkemann, 1940; Zeller, Stern & Wenk, 1941*a*), working independently, came to the same conclusion and emphasized that this activity is characteristic only of pregnancy and does not occur in any other condition. According to Werle & Effkemann (1940) and to Zeller (1938 *a*, *b*), the histaminase reaction in the blood becomes positive in the very early months of pregnancy, remains positive until delivery, and disappears within the first few days of the puerperium. From numerous experiments Zeller (1938 *a*, *b*), Zeller, Birkhäuser, Mislin & Wenk (1939*a*), Zeller, Schär & Staehlin (1939*b*) and Zeller, Stern & Wenk (1940) claim that the histaminase discovered by Best in 1929 is identical with the diaminoxidase which Zeller himself described in 1938 and which he found to be able to destroy not only histamine but also other naturally occurring diamines like cadaverine, putrescine, agmatine, spermin, etc. Zeller suggests that the diaminoxidase reaction is an oxidative one according to the equation

$$R\text{-}CH_2NH_2 + O_2 + H_2O = R\text{-}CHO + NH_3 + H_2O_2.$$

Evidence that H_2O_2 is probably formed by this system was given by Stevenson (1943), who investigated the mechanism of the histaminase reaction upon histamine, putrescine and cadaverine. The purpose of this work was not only to study the histaminase activity in the serum of patients with normal and toxaemic pregnancy but also to investigate the activity of this enzyme in the placenta and its possible significance in the onset and course of labour. Danforth (1937) and Zeller *et al.* (1939 *a*, *b*) report the occurrence of the histaminase in the human placenta. Although in Danforth's experiments the amount of histaminase in the placenta showed some correlation with the efficiency of uterine contractions, the author did not think that the evidence was sufficient to draw a definite conclusion.

EXPERIMENTAL

One of the chemical methods for the estimation of the activity of the diaminoxidase (histaminase) in the serum devised by Zeller, Stern & Wenk (1941*b*) is based upon the decoloration of a solution of indigo disulphonate by the H_2O_2 formed in the reaction between the diaminoxidase and its substrate. For this purpose Zeller advocates the use of cadaverine as substrate, since he found that histamine inhibits its own oxidation if present in superoptimal amounts. I have adopted this colorimetric method for my investigations after having made minor alterations in the amount of reagents. The required reagents are: (1) A solution of M/15 phosphate buffer (Sörensen) with pH 7·2. (2) A M/20 solution of cadaverine hydrochloride in M/15 phosphate buffer. (3) A solution of 20 mg. indigo disulphonate in 30 ml. of M/15 phosphate buffer.

Examination of serum. The serum is dialyzed against M/15 phosphate buffer in a refrigerator overnight. Haemolyzed sera must not be used.

To 3 ml. of the dialyzed serum is added 0·5 ml. of the M/20 cadaverine hydrochloride solution, and to 3 ml. of the control serum 0·5 ml. of the phosphate buffer solution is added. To each test-tube 0·2 ml. of the indigo disulphonate solution and 0·1 ml. of toluene are then added and oxygen is allowed to bubble through the solution for 1–2 min. The test-tubes are closed with a rubber stopper and incubated for 24 hr. at 37°. After that time the decoloration of the indigo disulphonate is examined. The initial blue colour of the control does not as a rule change, whereas the colour of the solution under assay has (*a*) completely disappeared or (*b*) diminished or (*c*) not changed at all. The estimation of the activity of the diaminoxidase (histaminase) is based upon the extent to which the indigo solution has been decoloured; thus + + + for complete decoloration, + + for distinct decoloration, + for slight decoloration, 0 for no decoloration.

Examination of placentae. 10 g. of the thoroughly ground placenta are extracted with 25 ml. of 2·5% (w/v) NaCl solution and the extract dialyzed against M/15 phosphate buffer solution in the refrigerator for 24 hr. The precipitate is then centrifuged off. To 2·5 ml. of the placental extract 0·5 ml. of the M/20 solution of the substrate is added and then to each test-tube 2 ml. of the indigo solution and 0·3 ml. of toluene are introduced. The rest of the procedure is the same as that described above for serum.

RESULTS

Sera from 101 pregnant and non-pregnant subjects have been examined. Eight sera of non-pregnant subjects gave a completely negative result. Twenty-three mixed sera of women at various stages of gestation (12–40 weeks) showed a vigorous enzymic activity (+ + to + + +). In five cases the addition of 5 mg. of aneurin to 3 ml. of serum brought about a complete inhibition of the enzymic activity: no decoloration of the indigo solution occurred. This confirms the observation of Zeller *et al.* (1939*b*) that solutions of the diaminoxidase were completely inactivated by the presence of aneurin. The results obtained in the investigation of the serum of six patients suffering from pre-eclamptic toxaemia were very surprising since, contrary to my expectation based on the previous work quoted above, they showed no diminished activity of the serum except in one case. Until then, following Zeller's work, I had measured the activity of the diaminoxidase (histaminase) by using only cadaverine as substrate. It became clear to me that, if any conclusion was to be drawn from the experimental work regarding histaminase metabolism in pregnancy, histamine had to be used as substrate. Therefore, it was decided to use M/20 solutions of histamine hydrochloride, as well as of cadaverine hydrochloride, as substrates in parallel tests.

The sera of nine non-pregnant women were first examined by this modified technique; the results obtained were completely negative. The investigation of 32 mixed sera from women at various stages of normal pregnancy (12–40 weeks) was then carried

out. As can be seen from Table 1 most sera showed the same enzymic activity with either substrate. In a few instances, however, the activity appeared to be slightly less with histamine as substrate. Added to five sera, aneurin inhibited the enzyme activity both when histamine and cadaverine were used as substrates.

Table 1. *Diaminoxidase activity of sera from normal pregnant women, with cadaverine and histamine as substrates*

(Activity estimated by degree of decoloration of indigo-disulphonate: + + + complete, + + distinct, + slight, 0 no decoloration.)

No. of patients	Weeks of gestation	Diaminoxidase activity substrate		Activity of diamin-oxidase in presence of aneurin
		Cadaverine	Histamine	
16	12–20	+ + to + + +	+ + to + + +	0
16	20–40	+ + to + + +	+ + to + + +	—

The results obtained in ten cases of mild pre-eclamptic toxaemia are presented in Table 2. While the results with cadaverine as substrate have been found to be on the whole comparable with those in normal pregnancy serum, this was not the case when histamine was used as substrate. In seven out of ten cases a distinctly diminished activity of the enzyme was found when the results were compared with the normal. Table 2 also shows results given

Table 2. *Diaminoxidase activity of sera from women with mild and severe pre-eclamptic toxaemia*

No. of patients	Weeks of gestation	Diaminoxidase activity substrate		
		Cadaverine	Histamine	
Mild pre-eclamptic toxaemia				
3	28–40	+ +	Trace	
1	40	+	Slight trace	
3	38–40	+ + to + + +	+	
3	34–37	+ + +	+ +	
Severe pre-eclamptic toxaemia				
4	31–35	+ to + +	0	
3	28–31	+ + to + + +	Trace	
1	32	+ + +	+	Still-birth
2	33	+ + +	+ +	Still-birth

by the sera of ten patients with severe pre-eclamptic toxaemia. In most of the sera there was again little difference in the enzymic activity with cadaverine as substrate as compared with that in normal pregnancy. When, however, histamine was used as substrate, completely different results were obtained. In four out of ten sera no activity was observed and in three others only a trace. In the sera of two patients, however, a normal activity of the diaminoxidase was found irrespective of whether cadaverine or histamine had been used as substrate. Both patients had stillbirths; intra-uterine death occurred several days before delivery and before the enzyme estimation had been carried out. The very serious condition of both patients cleared up also a few days before delivery.

Table 3. *Diaminoxidase activity of sera from women with eclampsia*

No. of patients	Weeks of gestation	Diaminoxidase activity substrate	
		Cadaverine	Histamine
2	36–40	+ + to + + +	0
1	37	+ +	Trace

Table 3 shows the results obtained with the sera of three eclamptic patients. Whereas enzymic activity comparable with that in normal pregnancy was found with cadaverine as substrate, no activity was seen in two sera and only a trace in one serum when histamine was used as substrate. In four out of five cases of *hyperemesis gravidarum* negative enzymic reactions were obtained in the serum irrespective of the substrate used. In one of the cases normal activity was obtained with cadaverine whereas only a trace of activity was seen with histamine as substrate. The sera of four pregnant patients, three of them suffering from hypertension and one from cardiac failure, gave normal results with both substrates.

Placentae from 45 women with normal and instrumental delivery were examined for histaminase activity, with cadaverine and histamine as substrates in parallel tests. Table 4 shows the results

Table 4. *Diaminoxidase activity of placentae from women with normal labour*

No. of patients	Diaminoxidase activity substrate	
	Cadaverine	Histamine
8	+ + to + + +	0
3	+ + to + + +	Trace
1	+ + +	+

obtained in the examination of twelve placentae from women with normal labour (with efficient uterine contractions). While enzymic activity was high in the experiments with cadaverine as substrate, the analogous experiments with histamine as substrate gave, in most cases, completely negative results. The results differed when placentae of eight women who had been delivered by caesarean section before the onset of labour were examined (Table 5); vigorous activity was found with both cadaverine and histamine as substrates.

In Table 6 are the results obtained on seven placentae from women who underwent caesarean section after a trial of labour. Whereas normal results

Table 5. *Diaminoxidase activity of placentae from women with caesarean section before the onset of labour*

No. of patients	Diaminoxidase activity substrate	
	Cadaverine	Histamine
1	+ + +	+ +
7	+ + +	+ + +

Table 6. *Diaminoxidase activity of placentae from women with caesarean section following trial of labour*

No. of patients	Diaminoxidase activity substrate	
	Cadaverine	Histamine
1	+ + +	+ + +
1	+ + +	+ +
3	+ + +	+
1	+ +	Trace
1	+ +	0

were again obtained with cadaverine as substrate, the findings with histamine were variable. With two out of seven placentae there were normal positive results, with three others a weak positive; one placenta showed a trace of enzymic activity and with another the enzymic reaction was negative.

Five placentae from women with prolonged labour were examined (Table 7). Strong activity was as usual observed with cadaverine as substrate. With histamine as substrate three placentae showed a strong enzymic reaction and two a weak one. Ten

Table 7. *Diaminoxidase activity of placentae from women with prolonged labour*

No. of patients	Diaminoxidase activity substrate	
	Cadaverine	Histamine
3	+ + +	+ + to + + +
2	+ + +	+

placentae from patients with pre-eclamptic toxaemia, who delivered themselves spontaneously, were examined. With cadaverine as substrate, vigorous enzymic activity was seen in all except one placenta; with histamine, no activity, or only a trace, was observed in all the placentae. In three cases of eclampsia cadaverine gave the usual strongly positive reaction; histamine, however, gave negative results, in two cases, and in one case showed only a trace of enzymic activity.

DISCUSSION

The results obtained in the investigations on pregnancy serum and on placentae make it clear that Zeller's claim that histaminase and diaminoxidase are identical should be thoroughly re-examined. The findings in the experiments with histamine and with cadaverine as substrates are on the whole so completely different that the question arises whether the histaminase really is identical with the diaminoxidase.

As to the results obtained with the sera of non-pregnant women and of those with normal pregnancy, the conclusions of previous authors are confirmed. The histaminase reaction was found to be negative in the sera of non-pregnant individuals, and positive without an exception in normal pregnancy serum. The results found in the sera of women suffering from toxaemia of pregnancy, when histamine was used as substrate. were significant. As was to be expected from previous work quoted above, diminished histaminase activity was seen in cases of mild pre-eclamptic toxaemia, and only a trace of activity or a negative enzymic reaction was obtained in cases of *hyperemesis gravidarum*, in severe cases of pre-eclamptic toxaemia and in eclampsia. The two cases of severe pre-eclamptic toxaemia, in both of which intra-uterine death had occurred and in the sera of which normal histaminase activity was found, are interesting so far as the results obtained may be suggestive of a possible restoration of a normal histaminase reaction in the serum caused by the death of the foetus. The completely normal findings in the cases of hypertension and cardiac failure may suggest that a diminished activity or absence of the histaminase in the serum may be characteristic only of toxaemic pregnancy. More investigations, however, must be carried out on this subject. The results obtained in the investigations on 45 placentae with respect to histaminase activity may suggest that the amount of histaminase in the placenta might be correlated with the character of the labour, for the activity of the histaminase in the placenta was found to be inversely proportional to the uterine efficiency. Little or no histaminase activity was found in the placentae of women who had had a normal labour with very efficient contractions, whereas high enzymic activity was seen in the placentae of patients who had had to undergo a caesarean section before the onset of labour. Accordingly, the placentae of women with prolonged labour and poor uterine contractions, and of patients with primary or secondary uterine inertia, contained varying amounts of histaminase. No spectacular conclusions can yet be drawn from the findings with the placentae of women with pre-eclamptic toxaemia and eclampsia. In view of previous work histaminase activity might be expected to be much diminished or even completely absent in these cases. As all patients, however, had a spontaneous delivery, the fact that no enzymic activity or only a trace of it has been found in the placentae has therefore no significance, since, as shown above, placentae of women with normal pregnancy and spontaneous delivery do not contain histaminase. More work, therefore, will have to be done on these lines.

Although these experiments are far from complete, it appears possible from the data obtained that histaminase might play an important part at the onset and during labour. It may be suggested that histaminase, which is very effective in the placenta during pregnancy, might become inhibited at the end of pregnancy by the action of some compounds, possibly the sex hormones, which are known to play an important role at the onset of labour. It may be recalled that in 1937 (Kapeller-Adler, 1937) it was shown that the gonadotropic hormones inhibit the activity of the histidase.

SUMMARY

1. The activity of the histaminase in the sera and placentae of pregnant women has been studied with two different substrates, cadaverine and histamine, in parallel tests.

2. The histaminase reaction is negative in the serum of non-pregnant women.

3. In normal pregnancy serum the histaminase test was found to be positive without exception, irrespective of the substrate used.

4. The activity of the histaminase is diminished in mild cases of pre-eclamptic toxaemia and only a trace of activity or a negative enzymic reaction was obtained in cases of *hyperemesis gravidarum*, in severe cases of pre-eclamptic toxaemia and in eclampsia.

5. The results obtained in investigations on 45 placentae suggest a possible correlation of the amount of the histaminase in the placenta with the character of the labour, the activity of the histaminase in the placenta having been found to be inversely proportional to the uterine efficiency.

6. The results obtained render doubtful Zeller's claim that the histaminase and diaminoxidase are identical.

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A Study of the Determination of Glucuronic Acid by the Naphthoresorcinol Reaction, with the Photoelectric Absorptiometer

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Methods for the determination of glucuronic acid in biological materials have appeared intermittently in the literature for the last 50 years. That none of these methods is entirely satisfactory is not surprising, since the reactions upon which they are based are not specific. The problem of the accurate determination of glucuronic acid in biological materials is therefore a difficult one.

Glucuronic acid may be estimated by the following methods: (1) those based upon measurement of its reducing properties, e.g. by Shaffer-Hartman and Bertrand's reagents (Goebel & Babers, 1933); by Benedict's reagent (Quick, 1924, 1925); by the ferricyanide reduction method of Miller & Van Slyke (Fishman, 1938); (2) those depending upon the formation of furfural by the action of hydrochloric acid on glucuronic acid (e.g. C. Tollens, 1909; Haendel, 1929; Tanabe, 1938); (3) those depending upon the carbon dioxide set free by the decarboxylation of glucuronic acid with mineral acids and other

reagents (B. Tollens & Lefèvre, 1892, 1907; Buston, 1932; Voss & Pfirschke, 1937; Freudenberg, Gudjons & Dumpert, 1941); (4) those depending on the violet colour produced when glucuronic acid is heated with naphthoresorcinol (1:3-dihydroxynaphthalene) and hydrochloric acid (B. Tollens & Rorive, 1908; B. Tollens, 1908). This reaction is given by other uronic acids, and pigments are also formed by sugars such as glucose, fructose and pentoses (cf. Neuberg & Kobel, 1931; Green & White, 1932).

Our purpose was to estimate the glucuronic acid output in urine after feeding 2:4:6-trinitrotoluene (α-T.N.T.) had been fed. Some of the results on T.N.T. have already been published (Channon, Mills & Williams, 1944). For pure solutions of glucuronic acid and conjugated glucuronic acids we found Fishman's method (1938) to be accurate and convenient. But for urine a ferricyanide method was useless, unless the glucuronide could be selectively extracted by a solvent, as in the case of menthylglucuronide. The method which seemed to offer the greatest possibilities was the one based on the Tollens naphthoresorcinol reaction (1908). An early attempt to put this colour reaction on a quantitative basis was made by C. Tollens (1909); and later the method was studied by Maughan, Evelyn & Brown (1938), by Kapp (1940), by Mozolowski (1940) and by Deichmann (1943) among others.

EXPERIMENTAL

In the methods of Maughan *et al.* (1938), of Kapp (1940) and of Deichmann (1943) ether was used to extract the pigment produced in the Tollens reaction. The intensity of the colour of the ethereal solution was then measured by means of a photoelectric colorimeter (Maughan *et al.*, and Deichmann) or a Stüfen photometer (Kapp). In the present work, colour intensity was measured with a Spekker photoelectric absorptiometer, but we found ether an inconveniently volatile solvent. Amyl ether was suitable but its purification is hazardous and it was therefore discarded. Of a number of alcohols tried, amyl alcohol proved satisfactory. Extraction of the pigment was rapid and complete, and the resulting solution had a blue colour with a sharp maximum absorption at 615–620mμ (see Fig. 1). The filter employed (Ilford no. 607 orange, one of the set of spectrum filters H. 558 issued by Adam Hilger, Ltd. for use with the absorptiometer) cuts off all light of wave-length less than 570mμ, thus removing the interfering absorption in the region of 510mμ when normal urine is used and at 480–505mμ when urine from T.N.T.-fed rabbits is used. The amyl alcohol solution of the pigment produced from both normal and T.N.T. urines showed a sharp maximum at 615–620mμ which is characteristic of that shown by the pigment from *l*-menthylglucuronide or glucurone (the curves from these compounds are almost identical (Fig. 1)).

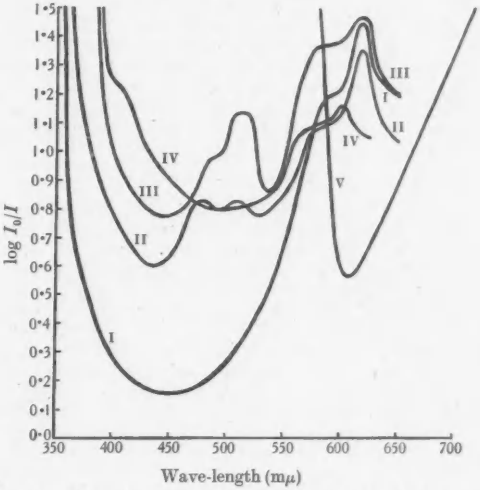

Fig. 1. Absorption curves of amyl alcohol extract of pigment from I, *l*-menthylglucuronide; II, T.N.T. urine (rabbit); III, normal urine (human); IV, *l*-menthylglucuronide in presence of an equimolecular amount of fructose; V, absorption curve of Ilford spectrum filter no. 607 (orange) reproduced from Ilford's catalogue (Ilford Colour Filters, p. 39).

Optimum conditions for colour production

Time of heating. In agreement with Kapp (1940) we found that 4 hr. in a boiling water-bath was necessary for maximum colour production. Over 90% of the colour had developed in 2 hr., and for economy of time this period of heating was adopted.

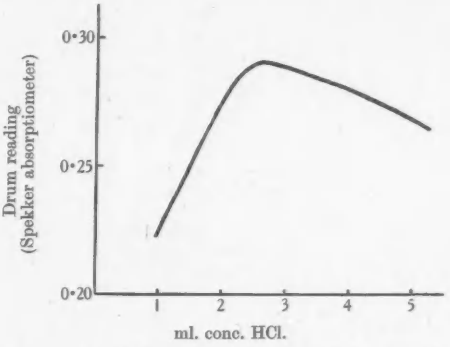

Fig. 2. Effect of acid concentration on colour production. 2 ml. solution plus 2 ml. 0·25% naphthoresorcinol plus variable quantity of conc. HCl.

Concentration of HCl. Maximum colour production is obtained when 3 vol. conc. HCl in a total of 7 vol. solution are used (see Fig. 2). Kapp (1940) found optimum colour production when the above ratio was 3 to 10.

Concentration of naphthoresorcinol. There is no sharp maximum for colour production on varying the naphthoresorcinol concentration. The colour intensity increased rapidly with rising concentrations to 0·25% solution and then only slowly for concentrations above this. Above 0·25% naphthoresorcinol, however, the blank value was significant and consequently a concentration of 0·25% was chosen.

Method of estimation

Reagents. Naphthoresorcinol (British Drug Houses, Ltd.), 0·25 g. dissolved in 100 ml. distilled water by keeping at 37° for 1 hr. The solution is filtered and stored at 0° in a dark bottle. It is stable for about 1 week. Hydrochloric acid, concentrated ('pure', J. W. Towers & Co. Ltd.). Amyl alcohol (Analar, British Drug Houses, Ltd.). Ethanol (absolute).

Procedure. 2 ml. of solution containing 10–80 μg. glucuronic acid, 2 ml. 0·25% naphthoresorcinol solution and 3 ml. conc. HCl in 6 × $\frac{5}{8}$ in. pyrex tubes, are mixed by rotation and placed in a boiling water-bath for 2 hr. The tubes are cooled in ice for 10 min., 5 ml. amyl alcohol added, and the tubes stoppered and shaken vigorously for 15 sec. The contents are poured into 50 ml. separating funnels, and washed out with a further 5 ml. amyl alcohol added to the separating funnels, the tubes being allowed to drain for 1 min. The aqueous layer is separated, the amyl alcohol solution run into graduated tubes, and the funnels allowed to drain for 1 min. The solution is made up to 11 ml. with ethanol (to dissolve any fine droplets of water suspended in the amyl alcohol), the contents of the tubes mixed and the colour estimated. A calibration curve was constructed for the Spekker photoelectric absorptiometer (10 mm. cells, Ilford 607 orange filters) with *l*-menthylglucuronide and glucurone. The curves for these two compounds were identical and the common curve is illustrated in Fig. 3.

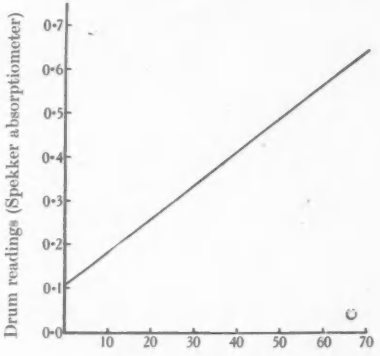

Fig. 3. Calibration curve for the estimation of glucuronic acid.

RESULTS

Typical figures for the recovery of glucuronic acid from pure solutions of various glucuronides are presented in Table 1. This table shows that most glucuronides can be estimated with an error of about ± 5%. Low results were obtained, however, with *o*-aminophenylglucuronide, *o*-amino-*p*-sulphonamidophenylglucuronide and euxanthic acid. The reason for these low results was investigated in the case of *o*-aminophenylglucuronide. This may be caused by either interference by the aglycone set free during hydrolysis or lack of hydrolysis of the glucuronide under the conditions of the estimation, i.e. heating for 2 hr. The first possibility was tested by estimating *l*-menthylglucuronide in the presence of an equimolecular proportion of *o*-aminophenol. The following typical results were obtained, which showed that this phenol had no appreciable effect on the estimation:

	Glucuronic acid	
Substances present	Calculated (μg.)	Found (μg.)
l-Menthylglucuronide alone	56·0	53·5, 54·5
l-Menthylglucuronide + *o*-aminophenol (33·6 μg.)	56·0	54·0, 52·5

The second possibility was tested by using longer periods of heating and comparing the rate of increase in colour produced by *l*-menthylglucuronide and *o*-aminophenylglucuronide after 2 hr. It was found that the rate of increase in colour between 2 and 4 hr. heating was greater for the latter substance than for the former (see Fig. 4 and Table 1). This result indicates that the low results for the *o*-amino-

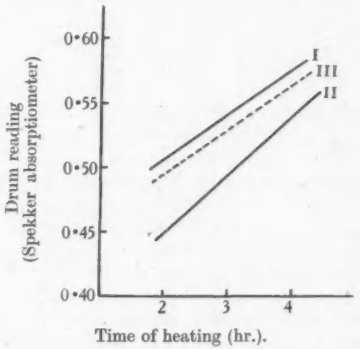

Fig. 4. Effect of increase of time of heating on colour production from *l*-menthylglucuronide (I) and *o*-aminophenylglucuronide (II). III is the theoretical curve for *o*-aminophenylglucuronide if it behaved in the same manner as *l*-menthylglucuronide.

phenylglucuronides are very probably due to incomplete hydrolysis under the standard conditions outlined above.

Table 1. *Recovery of glucuronic acid from pure solutions of glucuronides*

Glucuronide	Glucuronic acid: Calculated (μg.)	Glucuronic acid: Found (μg.)	
l-Menthylglucuronide	53·0	57·0,	56·0
*d-Iso*menthylglucuronide*	40·6	41·0,	—
p-Amino-*o*-sulphonamidophenyl-glucuronide†	31·0	32·5,	34·0
p-Aminophenylglucuronide†	44·9	47·5,	49·8
m-Aminophenylglucuronide†	51·0	53·0,	—
m-Aminophenylglucuronide†	48·0	48·8,	—
Sodium pregnandiolglucuronide‡	57·0	55·0,	—
o-Aminophenylglucuronide†	54·0	46·0, (51·0)‖,	45·0 (56·5)¶
o-Amino-*p*-sulphonamidophenyl-glucuronide†	31·3	22·5,	22·5
Euxanthic acid§	61·0	52·0,	52·6

* Williams (1938).
† Williams (1943).
‡ Venning (1938).
§ Euxanthone glucuronide, prepared from Indian yellow (Purree).
‖ After 3 hr. heating.
¶ After 4 hr. heating.

In the absence of interfering substances, added *l*-menthylglucuronide could be almost quantitatively recovered from normal human urine. The urine was diluted 1 in 50 before analysis and each estimation carried out in duplicate. Recoveries of 98–106 % were obtained. Similar results were obtained from normal rabbit urine.

Mucic acid and sugars such as glucose, fructose and pentose interfered. The interference was approximately equal for glucose and arabinose and slightly greater for fructose. Glucose, however, does not present a serious problem when present in amounts less than 50 μg. in 2 ml. of test solution, i.e. 2·5 mg./100 ml. The results in Table 2 illustrate this point. When more than about 50 μg. glucose is present the apparent glucuronic acid rises rapidly, even though the actual glucuronic acid remains constant.

Table 2. *Recovery of glucuronic acid in the presence of glucose*

Glucose added (μg.)	Glucuronic acid*: Added (μg.)	Glucuronic acid*: Found (μg.)
0	53·6	54·0
18	53·6	56·5
36	53·6	57·0
54	53·6	61·0

* Added as *l*-menthylglucuronide.

Since it may often be necessary to estimate glucuronic acid in the presence of glucose, the removal of the latter by fermentation was investigated (cf. Kakinuara, 1939, 1940). In our hands this method did not prove effective. The glucose content of the sample was reduced to 30–40 μg. by incubation with fresh washed brewer's yeast, but the results in Table 3 show that yeast contains water-soluble material, not completely removed by exhaustive washing with water, which produces a pigment when heated with HCl and naphthoresorcinol. This material appears in increasing amounts when the yeast is fermenting glucose. No fructose could be detected in the fermenting medium and the presence of small amounts of ethanol had little effect on colour production.

Table 3. *Effect of the products of fermentation of glucose on glucuronic acid estimation*

(Washed brewer's yeast suspended in 5 vol. tap water. Tubes incubated at 30° for 1 hr. and then centrifuged at 3000 r.p.m. for 5 min. 2 ml. solution used for each estimation in duplicate. Three experiments.)

Tube	*l*-Menthylglucuronic acid solution (ml.)	Water (ml.)	0·02 M-glucose (ml.)	Yeast suspension (ml.)	Glucuronic acid found: 1 (μg.)	2 (μg.)	3 (μg.)
A	1	2	0	0	45·0	42·0	50·0
B	1	0	1	1	63·7	59·0	85·5
C	1	1	0	1	57·0	54·0	63·5
D	0	1	1	1	33·0	36·0	53·5
Glucuronic acid present in tubes A, B and C					(46·6)	(41·6)	(52·0)

Glucuronic acid content of normal urine

The estimations were carried out as described above, with 2 ml. of urine diluted 1 in 50. The results showed (see Table 4) that adult laboratory workers on a normal British war-time diet excreted about 1 g. of glucuronic acid/day.

Table 4. *Glucuronic acid found in normal human urine*

Subject	Urine vol. in 24 hr. (ml.)	Glucuronic acid found: (g./day)	Glucuronic acid found: (mg./100 ml.)
1	1620	1·053	65·0
1	1070	0·976	91·6
2	1640	1·256	76·6
2	1114	0·929	83·3
2	1190	0·893	75·0
3	1530	1·325	86·6
3	1370	1·210	88·3
3	1295	0·757	58·3
Average	—	1·050	80·0

DISCUSSION

Perhaps the earliest attempt at estimating glucuronic acid in normal human urine was that made by Günther, de Chalmot & B. Tollens (1892). They evaporated 200 ml. of urine to dryness, carried out a furfural distillation on the residue, and obtained furfural corresponding to 40–50 mg. of glucuronic acid. If the daily excretion of urine is 1500 ml. their result corresponds to 300–375 mg. of glucuronic acid/day. Similar figures were obtained by C. Tollens & Stern (1910) both by the furfural distillation method and the colorimetric naphthoresorcinol reaction. Lower figures were obtained by Sauer (1930) (220–290 mg./day) and by Boku & Kin (1931) (13 mg./100 ml.). More recently values varying from 350–800 mg./day have been obtained by Maughan *et al.* (1938), by Wagreich, Kamin & Harrow (1940) and by Wagreich, Abrams & Harrow (1940), whilst Deichmann (1943) found the lower figures of 65–239 mg./day. All these recent values have been obtained by the Tollens colorimetric technique with the more modern photoelectric methods for measuring the colour, and it has been tacitly assumed that what is estimated is glucuronic acid, although there is no unequivocal proof of this.

It is claimed that the method developed in the present paper will estimate glucuronic acid added to normal urine with an accuracy of 5% in the absence of certain limiting proportions of sugars. On applying the method to the estimation of the natural glucuronic acid of urine we obtained figures higher than former workers (see Table 4). The material estimated in this way may or may not be entirely glucuronic acid. Eagle (1927) states that glucose is not normally excreted in urine and that what has been considered as glucose, a substance fermentable by yeast, is in reality a group of substances which only gradually decompose under the conditions of fermentation that remove glucose in a short time. However, if the average daily output of glucose in normal urine is 500 mg. and the daily urinary volume is 1500 ml., then 2 ml. of urine diluted 50 times will contain about 13 μg. of glucose. According to Table 2 this amount of glucose in 2 ml. solution does not interfere with the glucuronic acid estimation.

In the case of rabbits we have carried out a large number of estimations (see Channon *et al.* 1944). One group received a dry diet which produced a daily urine volume of *c.* 50 ml. In this group the range of glucuronic acid output was 52–218 mg./day with an average of 148 mg. Another group received a wet diet which gave a daily urine volume of *c.* 250 ml.; here the range was 62–196 mg./day and the average 140 mg.

It is known that a large number of substances excreted in normal urine are conjugated with glucuronic acid; these include phenol, *p*-cresol, indoxyl, sex hormones, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, catechol, to mention only a few. In addition to these there are present complex glucuronic acid-containing compounds such as urinary mucoid, urochrome and possibly mucopolysaccharides and unknown glucuronogenic substances of the diet. If the quantities of these were known it might then be possible to account for the colorimetric glucuronic acid values of normal urine.

SUMMARY

1. A study of the estimation of glucuronic acid by the Tollens naphthoresorcinol reaction is described. The relation of time of heating and of concentration of hydrochloric acid and of naphthoresorcinol to colour production has been studied, the intensity of colour in amyl alcohol being measured in the Spekker photoelectric absorptiometer with an orange filter.

2. Under the standard conditions described, glucuronides can be estimated with an accuracy of $\pm 5\%$ in the absence of certain interfering substances such as glucose and fructose. For certain glucuronides, however, the time of heating must be increased above the standard conditions adopted for routine estimations.

3. The method has been applied to urine and it has been found that the daily output of glucuronic acid is of the order of 1 g. for adult humans and 140–150 mg. for rabbits. The significance of these values is discussed.

We wish to express our grateful thanks to Dr H. J. Channon for his interest in the work and to Dr R. A. Morton for the absorption spectra.

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Carotene and Lycopene in Rose Hips and Other Fruits

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Rose hips have been previously reported to contain carotene (Kuhn & Grundmann, 1934; Wokes, Johnson, Organ & Jacoby, 1942) and lycopene (Karrer & Widmer, 1928; Escher, 1928), and they have also been found to possess considerable vitamin A activity (Svensson, 1936). Sufficient care has not always been taken to differentiate between the various carotenoid pigments. Moreover, data are needed on the amounts found in different species.

This paper provides such data by describing a simple method of estimating carotene and lycopene separately in the presence of other pigments, which has also given satisfactory results when applied to tomatoes and *Solanum Dulcamara* berries. The results on dried rose hip extract have been checked by a biological assay confirming the presence of vitamin A activity.

METHODS

Materials. Ripe rose hips of known species collected in the Durham area during October and November 1943 by Prof. Heslop Harrison, and in the Royal Botanic Gardens, Kew, during September 1943 by Dr R. Melville were posted immediately to King's Langley and stored in dry bottles in a refrigerator until examined; tests at intervals during the experimental period showed no loss of carotenoids under the given storage conditions. The samples of dehydrated rose hips were taken from large-scale batches prepared in the Ovaltine Laboratories in 1942–4 from locally grown hips, mainly *Rosa canina* and *R. dumetorum*. Ripe *Solanum Dulcamara* berries were collected in the King's Langley area during October 1943 and examined immediately. Ripe tomatoes grown out of doors in King's Langley in 1943 were preserved in jars by the usual method and when opened for examination 3 months later were in excellent condition.

Extraction of carotenoids. For this we have used an excellent unpublished method for extracting carotene from plant material devised by Dr Vernon Booth, to whom we are greatly indebted for advice and help. It was applied to rose hips as follows:

About 5 g. typical rose hips were selected and weighed. The flesh was dissected from seeds, stalks, etc., divided into two portions and weighed. Tests showed that no significant loss of moisture took place during the dissection, so that results with the flesh could be correlated with those from whole fruits. Each portion of the flesh (*c.* 1 g.) was ground with quartz powder and a mixture of acetone and petrol ether (2:3 by volume). The supernatant yellow solution was decanted into a separating funnel and the residue ground with more acetone-petrol ether mixture until no more colour was extracted. (With dried extracts and other dry material a few drops of distilled water were added to facilitate extraction.) The combined extracts were then washed with distilled water by a continuous flow apparatus, until the absence of striae as the drops of water fell through indicated that all acetone had been removed. Usually about a litre of water, passing through at the rate of about 2 drops per sec., was required to remove the acetone from 50 to 100 ml. of combined extract. The xanthophylls, which usually formed less than half of the total pigments, were removed by shaking two or three times with one-third volume of diacetonol until practically no further colour was removed. The diacetonol was then washed out with water.

Chromatographic separation. The adsorbent used in this investigation was Alocol brand colloidal aluminium hydroxide (A. Wander Ltd., King's Langley) activated by heating at 100° for a few minutes immediately before use. Its watery suspension had pH 7·4–7·5 as compared with 6·5–9·5 for other brands of alumina. The average particle size determined by means of haemocytometer was 25–100 μ, which is considerably higher than the 7 μ found by Zechmeister & Cholnoky (1941) for Merck alumina standardized by Brockmann's method. However, comparison with Merck and several other brands of alumina in use in this country at present has shown the Alocol after activation to be as efficient as any for separating carotene and lycopene. Recovery experiments with widely varying proportions of β-carotene and lycopene also gave satisfactory results. Details of this comparison will be published later.

The petrol ether extract, prepared as above, was percolated through a column of alumina (20 × 2 cm.). The pigments were adsorbed in a zone about 1 cm. deep and about

1 cm. from the top. The chromatogram was developed with petrol ether containing 1% acetone, when the yellowish orange carotene layer quickly separated from the reddish orange lycopene layer and, moving rapidly down the column, could be completely eluted. This usually left at the top of the column a narrow yellowish band possibly due to small amounts of kryptoxanthin. The lycopene was then eluted with benzene. The separate solutions of carotene and lycopene were stabilized with nitrogen, and stored in the dark in a refrigerator, under which conditions no appreciable loss occurred during several days. Throughout the extraction and separation of the pigments oxidation was prevented by keeping them moist with solvent and by screening them from light.

Spectrophotometric estimation of carotene and lycopene. This was done with a Hilger-Nutting constant deviation wave-length spectrophotometer calibrated for wave-length measurements against the emission spectrum of a helium lamp and for density measurements against a set of neutral glasses standardized by the National Physical Laboratory. The solutions, diluted if necessary with the respective solvents, were placed in 10 or 20 mm. cells of Hilger's type D, which can be readily filled and stoppered without inclusion of air bubbles in the body of the cell. The mean density was determined at 450 mμ for carotene in petrol ether (b.p. 40–60°) and 482 mμ for lycopene in benzene, using the cross-over technique of Wokes & Still (1942) which compensates for zero changes and other instrumental causes of error. Readings were taken at 10 mμ above and below the given wave-lengths to define the exact position of the maximum, and in a number of instances the whole curve was determined from 450 to 520 or 530 mμ. The results, summarized in Table 1, showed excellent agreement between carotene and lycopene separated from different sources and specimens of the pure pigments examined by ourselves and other workers.

Table 1. *Absorption data for carotene and lycopene*

	β-carotene		Lycopene	
Solvent	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1\,cm.}$	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1\,cm.}$
Petrol ether	450	2500	474*	2425
Benzene	463	2250	482	2025
Chloroform	463	2200*	480*	2000*

* Morton (1942).

Calculation of results. When calculating the concentration of carotene and of lycopene in our solutions we used the $E^{1\%}_{1\,cm.}$ values given in Table 1. The value of 2025 for lycopene in benzene was determined as follows. Lycopene was extracted from *Solanum Dulcamara* berries by the above method, and separated by chromatography from carotene and all other pigments. Solutions of the purified lycopene were prepared in chloroform, benzene and petrol ether (b.p. 40–60°) and their respective densities determined. From these densities were calculated the values for $E^{1\%}_{1\,cm.}$ in benzene and petrol ether, the value given by Morton (1942) for lycopene in chloroform being used as a basis. The benzene solution of the lycopene gave max. 482 mμ (between max. 486 mμ found by Zechmeister & Tuzson (1938) and max. 480 mμ found by Sivadjian (1938)) and the petrol ether solution gave max. 473 mμ, in good agreement with max. 474 mμ (Morton, 1942). The same technique applied to β-carotene in benzene gave $E^{1\%}_{1\,cm.}$ of 2250 at max. 463 mμ, differing by less than 2% from the value of 2280 at max. 464 mμ (Morton, 1943). Hence the error introduced by using the value 2025 for lycopene was probably less than 2%, of which only part could be attributed to the difference of 2–4 mμ in position of maxima as observed by ourselves and other workers, since the absorption curves for lycopene and carotene in Fig. 1 show fairly broad peaks at the above maxima.

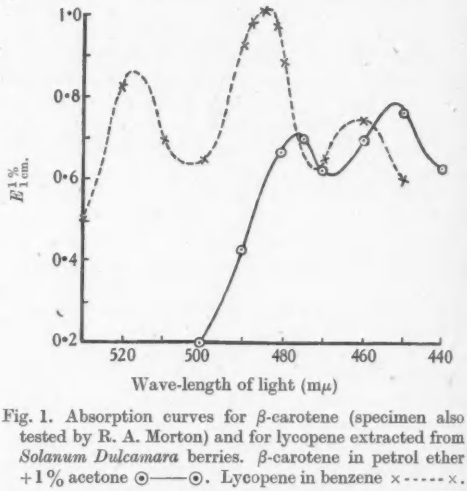

Fig. 1. Absorption curves for β-carotene (specimen also tested by R. A. Morton) and for lycopene extracted from *Solanum Dulcamara* berries. β-carotene in petrol ether +1% acetone ⊙——⊙. Lycopene in benzene ×-----×.

Sampling errors. These form the chief part of the experimental error. The average coefficient of variation for estimations on separate hips was 36. This was reduced to 15·7 by bulking 5 g. of hips comprising 3–12 hips, which after removal of seeds, etc., yielded 2–3 g. flesh. The average coefficient of variation between different 1 g. samples on the same lot of flesh from 5 g. portions of hips was only 5·1, showing that the method of preparing the samples had produced a much more uniform material than the original rose hip population. However, when dehydrated extracts of rose hips were made, the coefficient of variation between single assays on this material was still lower, showing that still greater uniformity had been obtained. Assuming that the experimental error was responsible for a coefficient of variation of not more than 3%, and deducting this from the average coefficient of variation of 36 for separate hips, there is left at least 33% as a measure of the variation to be expected from one rose hip to another.

RESULTS

Known species of rose hips. These are summarized in Tables 2 and 3 in order of decreasing carotene content. Each result is the mean of at least two assays in which the average error was as described above. The carotene content of the Durham samples was 74–187 μg./g., and of the Kew samples 41–671 μg./g. The lycopene content was usually higher than the carotene content, except in one or two

Table 2. *Carotene and lycopene in flesh of fresh rose hips from Durham*

	Carotene (μg./g.)	Lycopene (μg./g.)
Hybrid between *R. dumetorum* and *R. mollis*	187	276
R. dumetorum var. *Gabrielis*	161	232
R. dumalis var. *Reuteri*	149	532
R. dumalis var. *subcristata*	148	834
R. canina var. *sarmentacea*	146	268
R. tomentosa var. *scabriuscula*	142	141
R. obtusifolia var. *sclerophylla*	119	314
R. dumalis var. *subcanina*	118	229
R. coriifolia var. *frutetorum*	113	246
R. canina var. *vinacea*	98	223
R. dumetorum var. *pseudoincerta*	74	101

Note. We are informed by Prof. Heslop Harrison that in following the International Rules of Botanical Nomenclature, *R. dumalis* is the correct name to use for the species described as *R. glauca* or *R. Afzeliana* in Wolley-Dod's *Revision of the British Roses* (1930-1), the varietal names being the same except for var. *subcristata*, which is var. *glaucophylla* W.-Dod. The use of *R. dumalis* in this sense necessitates the change of *R. canina* var. *dumalis* to *R. canina* var. *sarmentacea*.

Table 3. *Carotene and lycopene in flesh of fresh rose hips from Kew*

	Carotene (μg./g.)	Lycopene (μg./g.)
R. centifolia	671	206
R. Davidii	242	657
R. carolina var. *Nuttalliana*	233	598
R. virginiana	230	327
R. moschata	210	147
R. calocarpa	175	150
R. Wilsoni	169	367
R. agrestis	123	537
R. Helenae	108	94
R. canina var. *stenocarpa*	100	204
R. Sabini	93	238
R. rubiginosa	74	595
R. mollis	63	283
R. spinosissima	50	146
R. coriifolia	41	354

species. The ratio between carotene and lycopene in a given species did not vary in duplicates by more than experimental error. The result of 187 ± 6 μg./g. for carotene in the hybrid of *Rosa mollis* and *R. dumetorum* was significantly higher than the results of 74 ± 1 for *R. dumetorum* hips collected at the same time in the same area, and higher than the result of $63 \pm 5{\cdot}0$ for *R. mollis* hips from Kew, although not much higher than the result of $161 \pm 11{\cdot}2$ for *R. dumetorum* var. *Gabrielis* hips from the Durham area. It should be noted that the hybrid was almost sterile, yielding less than a dozen hips on the whole bush.

Effect of latitude. Pyke & Melville (1942) found the vitamin C content of rose hips to be related to the latitude in which they are indigenous, the higher contents being found in higher latitudes. We did not find any positive relation between carotene content and latitude in native species showing marked differences in their vitamin C content (see Table 4). In fact our data suggested a negative correlation but were insufficient to establish this significantly.

Table 4. *Distribution of rose hips and their vitamin A and C values*

	Vitamin C* (mg./100 g.)	Carotene (μg./g.)
R. mollis	1260	63
R. coriifolia	1080	77
R. rubiginosa	810	74
R. tomentosa	690	142
R. dumetorum	590	118
R. canina	550	123
R. agrestis	460	123
R. obtusifolia	420	119
R. spinosissima	340	50

* Pyke & Melville (1942).

Comparison of carotene content with vitamin A activity. For this comparison it was thought desirable to obtain material of greater stability and more uniform composition than could be found in fresh rose hips. Dried extracts were therefore prepared from fresh ripe hips by extraction with boiling water and evaporation of the extract *in vacuo*, with precautions to avoid loss by oxidation. Tests showed that not more than 20 % of the vitamin C had been lost during the preparation of the extracts, which were found to be rich sources of vitamins C and P as well as containing considerable amounts of carotene. A biological assay of a sample of one of these dried extracts, after it had been stored about 9 months in powdered form in air at room temperature, was carried out in the Pharmaceutical Society's laboratories and showed it to have vitamin A activity equivalent to 44 i.u./g. (23–86 for $P = 0{\cdot}95$). Chemical estimation of the carotene content before and after the biological test showed a gradual loss of carotene (from 45 to 16 i.u./g. in 14 months) and the carotene content at the time of the biological test was only about 60 % of the amount shown by the biological test, if one assumes that 1 μg. carotene = 1·66 i.u. vitamin A. This rate of loss can be greatly reduced by storing the extracts in the form of compressed tablets. Taking into consideration the error of the biological method as well as other possible sources of error, this difference was not highly significant. However, since the biological result was higher than expected on the chemical assay indicated, it was clear that the carotene estimation did not provide too high an indication of the vitamin A value, and that the chromatographic separation of lycopene from carotene was complete.

Results on other materials. The method, having given satisfactory results with rose hips in which the L/C (lycopene/carotene) ratio ranged from 0·3 to 8·7, was then applied to other materials in which this ratio varied still more widely, e.g. *Solanum*

Dulcamara berries with L/C ratio of 17·2, dehydrated carrots rich in carotene and containing practically no lycopene, and tomatoes, which have in the past been given exaggerated vitamin A values because of failure to differentiate efficiently between carotene and lycopene. The results, summarized in Table 5, showed satisfactory separation of the carotene from the lycopene and were on the whole in good agreement with those obtained by other workers who also separated the two pigments satisfactorily (Kuhn & Grundmann, 1934).

Table 5. *Carotene and lycopene contents of different materials*

	Carotene (μg./g.)	Lycopene (μg./g.)
R. centifolia hips	671	206
Kew rose hips, 15 varieties, mean	172	327
Durham rose hips, 11 varieties, mean	132	309
Tomato skins, cooked, mean	73	416
R. coriifolia hips	41	354
Tomato flesh	7	33
Solanum Dulcamara berries	6	103
Results by other workers:		
Kuhn & Grundmann (1934):		
Tomatoes	7	79
R. canina hips	255	160
R. rubiginosa hips	230	275
Smith (1936):		
Tomato skins	2652–8862	—

In the skins of cooked tomatoes the carotene content of a number of samples was very much lower than that found by Smith (1936) in the skins of American tomatoes. The lycopene/carotene ratio in the skins was, however, similar to that found in tomato flesh by ourselves and by Kuhn & Grundmann (1934).

DISCUSSION

The estimation of carotene in materials containing lycopene presents difficulties. Failure to achieve efficient separation may lead to exaggerated claims for the vitamin A value of certain foods (e.g. tomatoes) if these claims are based on carotene estimations. Moreover, during the extraction of the pigments changes may occur through isomerization or oxidation. These difficulties have been overcome by using (*a*) extraction methods avoiding the use of heat and requiring minimum of exposure to air and light; (*b*) chromatographic separation with a specially prepared adsorbent of proved efficiency; (*c*) immediate spectrophotometric estimation of the pigments.

These precautions have enabled us to obtain reasonably accurate results on the different species of rose hips as shown by the coefficient of variation of 5·1 for different samples from the same batch of minced rose hip flesh.

It should be emphasized that this degree of accuracy is applicable only to the experimental material as we obtained it, and does not take into consideration variations caused by habitat, climate and other factors. However, since the average figure for all the Kew samples varied by less than 30 % from the average figure for all the Durham samples, the differences in conditions in these two areas could not account for the much wider variation in carotene and lycopene content in different species. Moreover, in a series of dried extracts made mainly from *Rosa canina* and *R. dumetorum* hips collected in the King's Langley area, there was less than 30 % deviation from one batch to another and from 1942 to 1943 seasons. Hence there is little doubt that ripe rose hips in general contain considerable amounts of carotene, and that some species contain much more than others.

SUMMARY

1. A method is described for extracting, separating, and estimating carotene and lycopene from plant materials, which minimizes isomerization and oxidative changes.

2. Applied to the flesh of ripe rose hips, this method showed carotene contents of 41–671 μg./g. and lycopene contents of 94–834 μg./g. in 26 distinct species and hybrids.

3. The method also gave satisfactory results with dried rose hip extracts, *Solanum Dulcamara* berries, tomatoes and other sources of carotenoids. The vitamin A value of a sample of dried rose hip extract as determined by biological assay was at least as high as was indicated by our chemical estimations of the carotene.

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